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**Degree:** Doctor of Philosophy

**Year This Degree Granted:** 2002

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**University of Alberta**

Mechanisms of Parenteral Nutrition Effects on Drug Metabolism

by

Yi Jenny Zheng



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment  
of the requirements for the degree of Doctor of Philosophy

in

Pharmaceutical Sciences (Pharmacokinetics)

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Spring 2002

**University of Alberta**



**University of Alberta**

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled “Mechanisms of Parenteral Nutrition Effects on Drug Metabolism” submitted by Yi Jenny Zheng in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences (Pharmacokinetics)



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## Abstract

Although its benefits, parenteral nutrition (PN) related complications have been reported. Studies showed that PN could alter cytochrome P450 (CYP) activity. One of the possible mechanisms is through cytokine and nitrite release that is triggered by endotoxin. The purpose of this study is to investigate the potential release of endotoxin, cytokines and nitrite during PN, and also to explore the impact of PN on individual CYP isozymes both *in vitro* and *in vivo*.

Rats were randomly assigned into either a) the PN group, which received continuous PN infusion only; or (b) the control group, which received normal chow with saline infusion. The infusions were continuous for 7 days and blood was collected and microsomes were prepared from the excised livers at the end.

Endotoxin levels in PN group were significantly higher in portal vein, but not in inferior vena cava when compared to those of the controls. TNF- $\alpha$  and IL-6 levels were significantly higher in the PN group ( $p < 0.05$ ). However, IL-1 $\beta$  levels were not significantly different in the two groups ( $p > 0.05$ ). The nitrite levels, the end product of nitric oxide formation, were found to be almost two times higher after PN ( $p < 0.05$ ).

PN also significantly reduced the protein and CYP content in rat liver microsomes. However, the impacts of PN on the individual isozymes were not uniform. It significantly decreased expression and activities of CYP2C11, 2A1/2, 3A2, but not CYP 2B1/2 and 1A1/2.

In an *in vivo* study, the half-life of midazolam, a model drug to evaluate CYP 3A isoforms in rat and human, was prolonged in rats treated with PN. The clearance of midazolam was reduced approximately 40% when compared to that of the control group.



In conclusion, it is confirmed that PN could lead to a reduction of liver CYP activities in rat to various degrees and result in a significant alteration of pharmacokinetic profile of certain drugs, such as midazolam. The changes in CYP activities were at least, in part, related to bacteria translocation that brought onto the increased levels of endotoxin. This increase triggered cytokine release, which down regulated the CYP activities (346 words).



## **Acknowledgment**

I wish to express my deepest gratitude to Dr. Yun K. Tam for his supervision, encouragement and patience throughout this project.

My sincere thanks also go to Dr. Ronald T. Coutts for his critical comments and valuable suggestions in editing my dissertation.

I wish to thank Drs. Soheir Tawfik, JJ Jiang, Ming-sheng Ma, Xing-fang Li deeply for their expert technical assistance during course of the study.

I am also very grateful to Dr. Nick Nation of Department of Laboratory Medicine and Pathology, University of Alberta for his help in conducting the liver histological examination and interpretations for the study.

Finally, I would like to thank the Faculty of Pharmacy and Pharmaceutical Sciences for providing a comfortable research environment and Canadian Medical Research Council for their financial support.



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## Abbreviations

$^{\circ}\text{C}$	Degree Celsius
APS	Ammonium persulfate
AUC	Area under the plasma concentration-time curve
BSA	Bovine serum albumin
CA	Carotid artery
$\text{CL}_{\text{total}}$	Total body clearance
cm	Centimeter (s)
$\text{C}_{\text{max}}$	Peak concentration
CV	Coefficient variation
CYP	Cytochrome P450
eNOS	Endothelial NOS
g	Gram(s)
<i>g</i>	G-force (acceleration of gravity)
GABA	$\gamma$ -aminobutyric acid
GI	Gastrointestine
HPLC	High-performance liquid chromatography
hr	Hour(s)
ICU	Intensive care unit
IL-1,6,8,10,12	Interleukin-1,6,8,10,12
iNOS	Inducible NOS
<i>iv</i>	Intravenous
IVC	Inferior vena cava
JV	Jugular vein
kg	Kilogram(s)
$\text{K}_{\text{m}}$	The concentration required to reach the half of maximum velocity ( $\text{V}_{\text{max}}$ )
L	Litre(s)
LAL	Limulus ameocyte lysate
LFT	Liver function test
LPS	Lipopolysaccharides
M	Molar(s)
MDZ	Midazolam
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)
mM	Millimolar(s)
mm	Millimeter(s)
mmol	Millimol(s)
MW	Molecular weight
n	Number of subjects studied
NADPH	$\beta$ -nicotinamide adenine dinucleotide phosphate
ng	Nanogram(s)



nM	Nanomolar(s)
nm	Nanometer(s)
nNOS	Neural NOS
NO	Nitric oxide
NOS	Nitric oxide synthase
PAPS	Phosphoadenosine phosphosulfate
PBS	Phosphate buffer solution
PCN	Pregnenolone-16 $\alpha$ -carbonitrile
PEG	Polyethylene glycol
pg	Picogram(s)
PN	Parenteral nutrition
PV	Portal vein
r	Coefficient of correlation
SDS	Sodium dodecyl sulphate
SOP	Standard operation procedure
T <sub>1/2<math>\alpha</math></sub>	Distribution half-life
T <sub>1/2<math>\beta</math></sub>	Elimination half-life
T <sub>c</sub> cell	T cytotoxic cell
TEMED	N,N,N',N'-tetramethylethylenediamine
T <sub>H</sub> cell	T helper cell
TLC	Thin-layer chromatography
TNF- $\alpha$	Tumor necrosis factor - $\alpha$
U	Unit(s)
V <sub>max</sub>	Maximum of velocity of metabolite formation
volt	Voltage(s)
V <sub>ss</sub>	Volume distribution at steady-state
x	Time(s)
%	Percentage(s)
N	Normal
$\mu$ g	Microgram(s)
$\mu$ l	Microliter(s)
$\mu$ m	Micrometer (s)
$\mu$ M	Micromolar (s)



# **1 Introduction**

## **1.1 Parenteral Nutrition (PN)**

### **1.1.1 Introduction**

For thousands of years the only route available for feeding was enteral; however, the whole area of nutritional support was revolutionized by Dr. Durick when PN was introduced in 1968 (Durick et al 1968). At the beginning, PN was a method of providing nutritional support for patients whose gastrointestinal (GI) tract was inaccessible or non-functioning. Later, PN became a standard practice for supplying energy and nutrition to critically ill patients and to those with severe chronic GI diseases. PN involves the delivery of nutrition directly into the circulatory system *via* either central or peripheral veins. The aim for PN is to supply the most comprehensive range of nutrients customized to the patient's need. The main component of PN solution is water. Energy requirements are met with glucose and fat solutions. Protein requirement is achieved using solutions of essential amino acids, measured in grams of nitrogen. Electrolytes, minerals, vitamins and trace elements are all included as required. In general, the indications for PN have included bowel obstructions, pancreatitis, perioperative nutritional support, GI bleeding and severe sepsis in the short term, as well as enteral feeding intolerance such as in short bowel syndrome or Crohn's disease in the long term. When used appropriately and with close monitoring, PN is a safe and effective method of providing nutritional support.

However, PN is not without side effects, particularly on the gut and liver. Examples are reduction of intestinal enzyme content and activity and inhibition of gut hormone production (Raftogianis et al 1995). Decreased mucosal wall thickness



associated with chronic PN is also accompanied by an increase in intestinal bacterial translocation across the gut into the systemic circulation (Alverdy et al 1988). Hepatic effects include steatosis and cholestasis (Klein & Nealon 1988; Raftogianis et al 1995), associated with a gradual increase in liver transaminases, alkaline phosphatase, and total bilirubin (Clarke et al 1991). In rare instances, long-term PN has lead to cirrhosis. Research has also demonstrated that PN increases infectious complications in critically ill and injured patients.

### **1.1.2 Influence of PN on Drug Pharmacokinetics**

Nutrition-drug interaction may alter pharmacokinetics (Roe 1985). However the effects of PN on drug disposition have not been well studied. PN potentially can affect the major processes of drug disposition, e.g. absorption, distribution, metabolism and elimination. Theoretically, the use of PN may change the stability and pharmacokinetic profile of drugs in a number of ways. For example, physiochemical conditions in a PN solution such as pH and concentration of the nutrients are important in the determination of drug stability *in vitro* (Colding & Andersen 1978). However, there are limited data on the specific role of nutrient regimens on pharmacokinetics.

#### **1.1.2.1 Absorption**

There is little data available showing direct evidence for altered drug absorption during administration of PN, but the morphologic and functional changes that occur in the GI tract during PN administration (Masclee et al 1996) potentially could alter drug absorption. However, PN is often used when there is an underlying dysfunction of the gastrointestinal tract. This limits the number of drug that can be given orally, so many



medication are given parenterally to critically ill patients receiving PN. The possibility of interaction must be considered when drugs are either directly added to or coinfused with the PN solution.

#### **1.1.2.2 Distribution**

Fat emulsion has been shown to alter the binding characteristics of drugs to serum proteins. The increase in free fatty acid concentrations with lipid administration has been reported to displace highly protein bound drugs, including morphine, clofibrate, valproic acid, salicylic acid and phenytoin (Rudman et al 1971; Crouthamel & Cenedella 1975; Zimmerman et al 1981). However, the distribution of low protein bound drugs such as ampicillin is unlikely to be affected to a significant extent with PN infusion (Koo et al 1990). The clinical significance of this interaction has not been adequately studied.

#### **1.1.2.3 Metabolism and Elimination**

Liver oxidative metabolism reflects the activity of the cytochrome P450 (CYP) enzyme system located in the liver microsomal fraction. This enzymatic system plays a major role in the metabolism of numerous endogenous and exogenous compounds. Microsomal enzyme activities and concentrations can be influenced by drugs (Brosen 1990; Watkins 1990; Murray 1992), disease status (Wright & Morgan 1991; Chen et al 1992), age (Waxman et al 1985), gender (Waxman 1984; McClellan-Green et al 1989), and diet (Knodell et al 1980; Ross et al 1983; Knodell et al 1984; Ross et al 1984; Knodell et al 1989; Knodell et al 1990). Both animal (Knodell et al 1984; Knodell et al 1989) and human (Quigley et al 1993) experiments show that hepatic CYP-mediated



oxidation is significantly impaired in rats or patients receiving continuous PN compared with control subjects receiving the same nutrients by the enteral route.

Ross et al. (1983) studied the effect of PN on the enzymatic activity of the liver by using antipyrine as a model drug. They found after seven days of continuous infusion with PN containing 25% glucose and 4.5% amino acids, antipyrine clearance was 73% lower than that seen in the control group of rats. This result is consistent with another study performed by Knodell et al (1984). They found that continuous *iv* administration of PN *via* the jugular vein produced a 25% lower microsomal CYP content. There was associated with a 41% decrease in meperidine demethylase activity, and a 55% decrease in pentobarbital hydroxylase activity in rats compared with control animals fed with an identical diet by the enteral route. However, this effect could be corrected by switching 30% of the nonprotein calories to fats in the PN solution (Ross et al 1984), suggesting that quantitative and/or qualitative differences in nutrition and energy sources may be responsible for differences in hepatic microsomal mixed-function oxidases activities observed between PN and control animals.

To determine whether the caloric source of intravenous nutrition can influence oxidative drug metabolizing capacity in human, the Pantuck et al (1984) studied antipyrine metabolism in six healthy volunteers. Subjects were taking an intravenous nutritional regimen of 5% dextrose, 440 kcal/day, for 4 days, and after that the dextrose infusion was replaced by a 3.5% crystalline amino acid solution (480 kcal/day) for one day. Drug clearance was increased by an average 24% with a range between 2-71% during the amino acid regimen. This suggested that amino acids, as high protein diets, given intravenously could accelerate drug oxidation in humans. But the wide range (2-



71%) of increase indicated there was marked variability in the responsiveness of different subjects to the change in intravenous caloric source. The same investigators (Pantuck et al 1989) also examined the effects of changing PN solution isocalorically from intravenous dextrose to amino acids (23%) on oxidative drug metabolism in eight healthy volunteers. The results showed that the rate of antipyrine clearance was increased by 22% with the amino acid regimen, which was in reasonable agreement with their previous study results. This study confirms that the level of amino acids influences antipyrine metabolism and the effect occurs without changes in the amount of calories received.

The effect of different PN regimens on hepatic oxidative function was also assessed by Burgess et al. (Burgess et al 1987) in postoperative patients receiving PN for 7 days. The control group received 4% dextrose and saline, providing 340 kcal/day. The first two experimental groups received dextrose as the only caloric source providing 1600 and 2000 kcal/day, respectively. The last group of patients received 2000 kcal/day of PN consisting of 75% dextrose and 25% lipid. The results showed that the groups receiving carbohydrates as the sole caloric source had a significantly lower rate of antipyrine clearance and the amount of calories provided has no effects. Antipyrine clearance was unchanged, however, in patients receiving PN in which lipid emulsion was substituted for some of the carbohydrate calories, suggesting that lipids might improve protein synthesis and reduce liver damage (Pomposelli et al 1986; Baldermann et al 1991).

The changes in oxidative metabolism induced by PN appear to be isozyme specific. Knodell and coworkers have also reported that continuous PN *via* the jugular vein produces selective effects on hepatic CYP (Knodell et al 1989). Continuous PN treatment in rats significantly reduced two hepatic constitutive forms of P450, namely



CYP 3A1/2 and 2C11 compared with control animals receiving the same diet by the enteral route. Since the amounts of hepatic CYP 2A1 and 2C6 were unchanged, this result suggested that PN elicited selective effects on gene transcription.

Phase II reactions, such as glucuronidation and sulfation, involve metabolic conversion of a wide variety of endogenous and exogenous chemicals. The rate of drug conjugation depends on both enzyme activity and on the availability of the endogenous co-substrates, such as UDP-glucuronic acid and 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Alterations in conjugation reactions can affect the metabolic fate and toxicity of a drug.

One study examined the effect of PN on hepatic conjugative capacity and found that 5 days of parenteral infusion of amino acid and glucose resulted in a small (~13%) but statistically significant decrease in bilirubin conjugation in liver homogenates (Culebras et al 1993). Raftogianis and coworkers (Raftogianis et al 1995) reported that an infusion of PN for longer than 10 days markedly decreased hepatic conjugative metabolism. The clearance of acetaminophen, which is metabolized largely *via* conjugation, decreased by 23%.

It is quite clear that the small intestine is an important site of drug metabolism. Both cytochrome P-450 and glutathione S-transferases, have been identified in rat small intestine (Chhabra et al 1974; Pinkus & Windmueller 1977). The intestinal drug metabolic activity could also be affected by a variety of changes in intestinal morphological and function in response to continuous PN infusion. Knodell et al. (Knodell et al 1980) assessed the differences in intestinal pentobarbital metabolism between parenterally or enterally nourished rats after seven days of continuous



administration of an amino acid-glucose mixture *via* a gastric or jugular vein catheter. After giving pentobarbital orally, higher plasma concentrations and greater area under the curve values were achieved in the parenteral group than in the enteral animals. The authors claimed this resulted from decreased intestinal metabolism of pentobarbital in the parenterally nourished animals due to the impairment of gut enzyme capacity. Other contribution factors, such as absorption, should also be taken into account because it is also well known that PN infusion can change the morphology of the intestine.

As was mentioned before, most patients who need PN are unable to maintain a nutritionally balance diet due to an inaccessible or non-functional gastrointestinal tract. These patients usually receive medications parenterally. Therefore, the clinical significance PN effects on gut metabolism is limited.

## **1.2 Endotoxin**

### **1.2.1 Properties of Endotoxin**

Endotoxins are large (molecular weight, 200,000 to 1,000,000), heat-stable (to 100 °C) lipopolysaccharides (LPS), which are the major components of the cell wall of gram-negative bacteria. Each consists of a carbohydrate core, the lipid A region and a polysaccharide O antigen region. An endotoxin induces multiple biological effects *in vivo*, including fever, leukocytosis, hypoferrremia, platelet aggregation, thrombocytopenia (Morrison & Ulevitch 1978; McCabe 1980; McCartney & Wardlaw 1985). The effects can be attributed to activation of various endogenous pathways or triggering a cascade reaction. For example, LPS triggers coagulation, fibrinolytic, and kinin pathways to release cytokine mediators from macrophages and monocytes. The release of these



mediators in turn triggers the characteristic biological effects induced by cytokines. Nearly all of these effects are mediated through the lipid A region.

### **1.2.2 Limulus Amebocyte Lysate (LAL) Assay**

The level of endotoxin as measured using the LAL assay closely parallels the density of bacteria throughout logarithmic growth. Levin and coworkers showed that an endotoxin-initiated reaction could cause the enzymatic conversion of a clottable protein derived from the circulating blood cell (amebocyte) of the crab (Levin & Bang 1968; Young et al 1972). They recognized the potential for this biological reagent as a diagnostic tool and characterized its properties. Several LAL assays have been developed since then. Examples are gel clot, coagulogen-based, and chromogenic LAL assays. In the current study, we used the chromogenic LAL assay to quantify endotoxin. The chromogenic LAL assay has two stages: a LAL activation stage, followed by a chromophore release stage, which is triggered by the addition of the chromogenic substrate to the reaction mixture. The release of chromophore imparts a yellow color to the solution. The strength of the yellow color (measured as optical density at 405 nm in a spectrophotometer) is a function of the amount of active clotting enzyme (and indirectly to the amount of endotoxin) present in the solution. Both phases of the chromogenic reaction are critically time and temperature dependent, but within these limitations the chromogenic assay is sensitive up to 10 pg/ml (Thomas et al 1981).

### **1.2.3 Endotoxin Clearance**

Bacteria endotoxin is known to interact with numerous component of blood, including erythrocytes, mononuclear cells, platelets, neutrophils, lipoproteins, and plasma



proteins (Roth et al 1993). Clearance of endotoxin is effected both by humoral inactivation and uptake into the liver and mononuclear phagocytic cells (Das et al 1973; Skarnes 1985; Freudenberg et al 1992) and this elimination process is also influenced by both host- and LPS-specific factors. Binding of endotoxin by chylomicrons probably facilitates the clearance of endotoxin through the liver (Read et al 1993). The clearance patterns (Ge et al 1994) and biological effects (Cross et al 1993) following administration of live bacteria are different from those following the administration of an equivalent dose of purified endotoxin.

In patients with sepsis, endotoxin is typically at a level as high as 400 pg/ml or higher. In patients without sepsis endotoxin level can reach as high as 200 pg/ml. It is misleading to attempt to extrapolate from a level of endotoxin in a patient with sepsis to an exogenous dose of endotoxin that would induce comparable pathophysiological abnormalities. Exposure to a bolus dose of endotoxin, as in “intoxication” experimental models, could result in a burst of cytokines production at high levels, and is in contrast to the low-grade ongoing cytokine production observed in experimental models of infection or even in clinically observed gram-negative sepsis (Cross et al 1993).

#### **1.2.4 Endotoxin and Liver Diseases**

Endotoxemia has long been suspected of having pathogenic properties in patients with liver disease even in the absence of gram-negative bacteria (Nolan 1975). The origin of the endotoxin in this setting is also believed to be from the gastrointestinal tract because several studies have found that a portal-to-systemic gradient of endotoxin levels, with higher levels in portal venous blood than in peripheral blood (Prytz et al 1976; Jacob et al 1977; Bigatello et al 1987; Lumsden et al 1988). There has been some success at



reducing endotoxemia by the oral administration of endotoxin-binding agent such as bile salts (Thompson et al 1986), colistin (Guarner et al 1993) and paromomycin (Tarao et al 1982). Some studies found an association between endotoxemia and abnormalities in routine biochemical liver function test (Bigatello et al 1987), whereas others have not (Prytz et al 1976; Fukui et al 1991; Kokuba et al 1993), although it should be noted that these tests are relatively insensitive indicators of liver dysfunction in comparison to histological evidence.

Bacterial translocation has been demonstrated in animal models and in humans under certain clinical conditions, such as bowel obstruction and septic shocks (Deitch et al 1990; van Goor et al 1994). Lichtman et al. has shown that the presence of increased colon flora in the small bowel causes significant hepatic inflammation and hepatocellular necrosis (Lichtman et al 1990). The investigators further showed that endotoxin could be absorbed from the intestinal lumen during injury (Lichtman 1991), and metronidazole and tetracycline could prevent this condition. Studies also showed that enzymatic degradation of endotoxin prevented hepatic injury caused by bacterial overgrowth in rats (Lichtman et al 1992). These findings implicate absorbed bacteria or bacterial cell wall components from the intestinal lumen may be a major causative factor in the pathogenesis of liver injury (Lichtman & Sartor 1991).

A growing body of evidence has demonstrated that in many patient populations the enteral route of nutrition has significant advantages over PN by keeping gut normal morphology and maintaining its regular immune system. Enteral feeding also decreases infectious complications in critically ill and injured patients. In a study (Kudsk et al 1983), rats were fed with identical PN solution either *via* gastrostomy (the enteral group)



or central catheter (the parenteral group) for 12 days before intraperitoneal hemoglobin-E *coli* injection. The 48-hr survival was 60% in the enteral group and 20% in the parenteral group. The authors explained that lack of normal gut stimulation during parenteral feeding impaired gut membrane, increased gut permeability, and subsequently allowed larger amounts of endotoxin enter the circulation, and led to the low survival rate in the parenteral group.

## 1.3 Cytokines

### 1.3.1 General Properties of Cytokines

Cytokines are a group of low-molecular-weight regulatory proteins secreted by white blood cells and a variety of other cells in the body in response to a number of inducing stimuli. Cytokines bind to specific receptors on the membrane of target cells, eliciting biochemical changes responsible for signal transduction that results in an altered pattern of gene expression in the target cells. Cytokines and their receptors exhibit very high affinity for each other with dissociation constants ranging from  $10^{-10}$  to  $10^{-12}$  M. Because of this high affinity, picomolar concentration of cytokines can mediate a biological effect.

A particular cytokine may exhibit *autocrine* action, binding to receptors on the membrane of the same cell that secret it. It may exhibit *paracrine* action, binding to a receptor on a target cell in close proximity to the producer cell. And in a few cases it may exhibit *endocrine* action, binding to target cells in distant parts of the body. Cytokines regulate the intensity and duration of the immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of various cells and by regulating their



secretion of antibodies or other cytokines. Binding a given cytokine to responsive target cells generally stimulates expression of cytokine receptors and of other cytokines, which in turn affect other target cells. Thus, cytokines secreted by a single lymphocyte following antigen-specific activation can influence the activity of various cells involved in the immune response. For example, cytokines produced by activated  $T_H$  cells can influence the activity of B cells,  $T_c$  cells, natural killer cells, macrophages, granulocytes, and hematopoietic stem cells, thereby activating an entire network of interacting cells.

Cytokines exhibit the attributes of pleiotropy, redundancy, synergy, and antagonism, which permit them to regulate cellular activity in a coordinated interactive way. A given cytokine that has different biological effects on different target cells has a *pleiotropic* action. Two or more cytokines that mediate similar functions are said to be *redundant*; this property makes it difficult to ascribe a particular activity to a single cytokine. Cytokine *synergism* occurs when the combined effect of two cytokines on cellular activity is greater than the additive effects of the individual cytokines. In some cases, cytokines exhibit *antagonism*, in which the effects of one cytokine inhibit or offset the effects of another cytokine. The term cytokine usually encompasses those cytokines secreted by lymphocytes, monocytes and macrophages.

Cytokines generally function as intercellular messenger molecules that evoke particular biological activities after binding to a receptor on a responsive target cell. The two major producers of cytokines are  $T_H$  cell and macrophage. The binding of a cytokine to its receptor induces a number of physiologic responses including the development of cellular and humoral immune responses, induction of inflammatory response, control of cellular proliferation and differentiation, and induction of wound healing. In addition,



cytokines often induce the synthesis of other cytokines, resulting in cascades of cytokine activity.

### 1.3.2 Cytokines and Diseases

Cytokines are essential mediators of the immune response and their role in several diseases, such as inflammation, cancer, burns, trauma, and sepsis, is crucial. *In vitro* studies with hepatocyte cell cultures, as well as *in vivo* studies, suggest that the primary mediators of the hepatic acute phase response include interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF- $\alpha$ ). Under *in vitro* conditions, IL-1, TNF- $\alpha$  and IL-6 decrease albumin synthesis, and, to a variable extent, increase the synthesis of acute phase proteins, but the pattern of protein synthesis in response to these cytokines varies depending on the cell line and the experimental conditions (Moldawer et al 1988). To define a specific tissue response to an individual cytokine is still impossible because considerable overlap and redundancy exists in the cytokine network. Under *in vivo* conditions, TNF- $\alpha$  can induce IL-1 (Dinarello et al 1986) and IL-6 biosynthesis (Van Zee et al 1994), whereas IL-1 can induce IL-6 (Fischer et al 1991). Thus, studies that employ pharmacological administration of recombinant cytokines frequently cannot discriminate between biologic responses induced directly by the administered cytokine, and those induced by mediators stimulated by that cytokine. Furthermore, inflammation often elicits a cytokine cascade in which several cytokines are induced simultaneously. For example, in primates or rodents with endotoxemia, bacteremia, or hemorrhagic shock, it is not uncommon to detect TNF- $\alpha$ , IL-1, IL-6, interleukine-8 (IL-8), interleukin-10 (IL-10), and interleukine-12 (IL-12) heterodimer simultaneously (Zhu et al 1994; Espat et al



1995). Under such conditions, it is difficult to resolve the contribution that each of these mediators is playing in the acute phase response.

The underlying mechanism of PN related liver injury is still unclear. It seems that PN may have various immunomodulating effects through either a direct effect on different immune indices, mainly cytokines, or a complex communication between nutrients and the immune response. Therefore, the effect of PN on the synthesis of different cytokines may have serious clinical implications. However, there are only a few clinical studies of the effect of PN on the production of some cytokines available in the literature.

Studies show that endotoxin could stimulate hepatic Kupffer cells to release pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1 and IL-6 (Lichtman et al 1994). Gogos et al. (1992) investigated the effect of PN duration on TNF- $\alpha$  production in peripheral mononuclear cells in human blood and found that long-term (>40 days) PN induced a significant increase in TNF- $\alpha$  production after endotoxin stimulation, whereas short-term PN did not. This seems to be important in diseases such as cancer and sepsis, in which TNF- $\alpha$  production might be deleterious.

Following a prolonged presence of an antigen, a chronic inflammatory response may develop. In a chronic inflammatory response, the accumulation of large amount of activated macrophages is responsible for the tissue damage. These cells released various hydrolytic enzymes and reactive oxygen and nitrogen intermediates resulting in damage to the surrounding tissue. One of the principal cytokines secreted by activated macrophages is TNF- $\alpha$ .



Bacterial septic shock appears to develop when bacterial cell-wall endotoxin stimulates macrophages to overproduce IL-1 and TNF- $\alpha$ . It is the increased levels of IL-1 and TNF- $\alpha$  that cause septic shock.

## **1.4 Free radical**

Free radicals, such as nitric oxide (NO) and superoxide, are defined as atoms or molecules with one or more unpaired electrons. They usually have very short half-life and are highly reactive. These highly reactive electrophilic chemical species can not only permanently damage cells by reacting with nucleic acids, proteins, and polyunsaturated lipids (lipid peroxidation), but may also lead to cell death. The stabilization of those unpaired electrons is accomplished by reacting with species, which also contain an unpaired electron, i.e. other free radicals. Reaction with scavengers, such as ascorbic acid (Vitamin C) can be protective, because the resulting species is no longer reactive (Beckman et al 1994).

The extremely short half-life makes direct measurement of free radicals especially difficult. This difficulty has lead to the development of many different methods for the measurement of free radical activity indirectly by their effects on biological systems, e.g. by measuring products of free radical attack on macromolecules, or by their effect on the antioxidant defense system (Knight 1995).

Owing to our increased understanding of these chemical species, a growing number of diseases/disorders have been linked either directly or indirectly with them. Some disorders are primarily due to free radicals, others may be only secondarily involved. In this latter group, tissue injured by various processes, such as trauma, toxic



substances, and infectious processes may undergo free radical damage more rapidly than healthy tissues.

#### **1.4.1 NO**

NO is formed from arginine through a specific synthesis by one of three NO synthase (NOS) isoenzymes, which are encoded by separate genes (Knowles & Moncada 1994). Low levels of NO are formed by endothelial NOS (eNOS) and neuronal NOS (nNOS). Both are constitutively expressed and the NO formed plays a variety of roles in cell signaling. eNOS is found primarily in vascular endothelial cells, where it plays an essential role in control of blood flow and blood pressure. nNOS is found in the brain and peripheral nervous system and has a role in memory formation. The third calcium-independent form of inducible NO synthase (iNOS) is not usually expressed under normal physiological conditions, but following induction, iNOS is capable of yielding high, cytotoxic levels of NO. iNOS can be induced in most mammalian cells studied to date and is expressed in response to bacterial products and cytokines as a component of the host-specific response to infection. The strongest evidence for this is in experimental *Leishmania* infection (Wei et al 1995). There is evidence for a pathological role of NO in human diseases such as multiple sclerosis (Bagasra et al 1995), atherosclerosis (Buttery et al 1996), and in carcinogenesis linked to chronic infection (Ohshima & Bartsch 1994; Tamir & Tannenbaum 1996).

NO has a reported half-life of between 5-15 seconds. Under normal physiological condition, its concentration is usually lower than 100nM and could become as high as 10μM at pathological status. NO is also a signaling molecule and exposure of cells to it can result in up- and down-regulation of a variety of gene products (Szabo 1996).



### **1.4.2 Free Radicals and Diseases**

An increase in the formation of free radicals has been observed in a number of different disease states. These include the formation of atheroma (Rice-Evans & Gopinathan 1995), inflammatory disorders (such as rheumatoid arthritis) (Taraza et al 1997), and infections and sepsis (Novelli 1997). Free radical production occurs during the engulfment and destruction of bacteria by white cells, during ischemia-reperfusion episodes and during the metabolism of arachidonic acid to prostaglandin and leukotrienes (Lunec 1990).

Because free radicals are known to be mediators in the pathogenesis of various disorders, it is possible that their level of activity may serve as a marker for the severity of the illness. Evidence of free radical activity has been correlated with an adverse outcome in very low birth weight infants (Inder et al 1996). An increase in the formation of free radical has also been found in neonates receiving total PN and has been linked to the administration of fat (Pitkanen et al 1991).

## **1.5 Testosterone**

CYP isozymes are responsible for most of the NADPH-dependent oxidation of endogenous compounds and xenobiotics. They play a role in activating and detoxifying xenobiotics. Given the unique catalytic functions ascribed to CYP enzymes, there is an ongoing need for enzymatic assays that provide specificity for the analysis of individual enzyme activity. It is well established that regio-, and stereo-specific hydroxylation of the



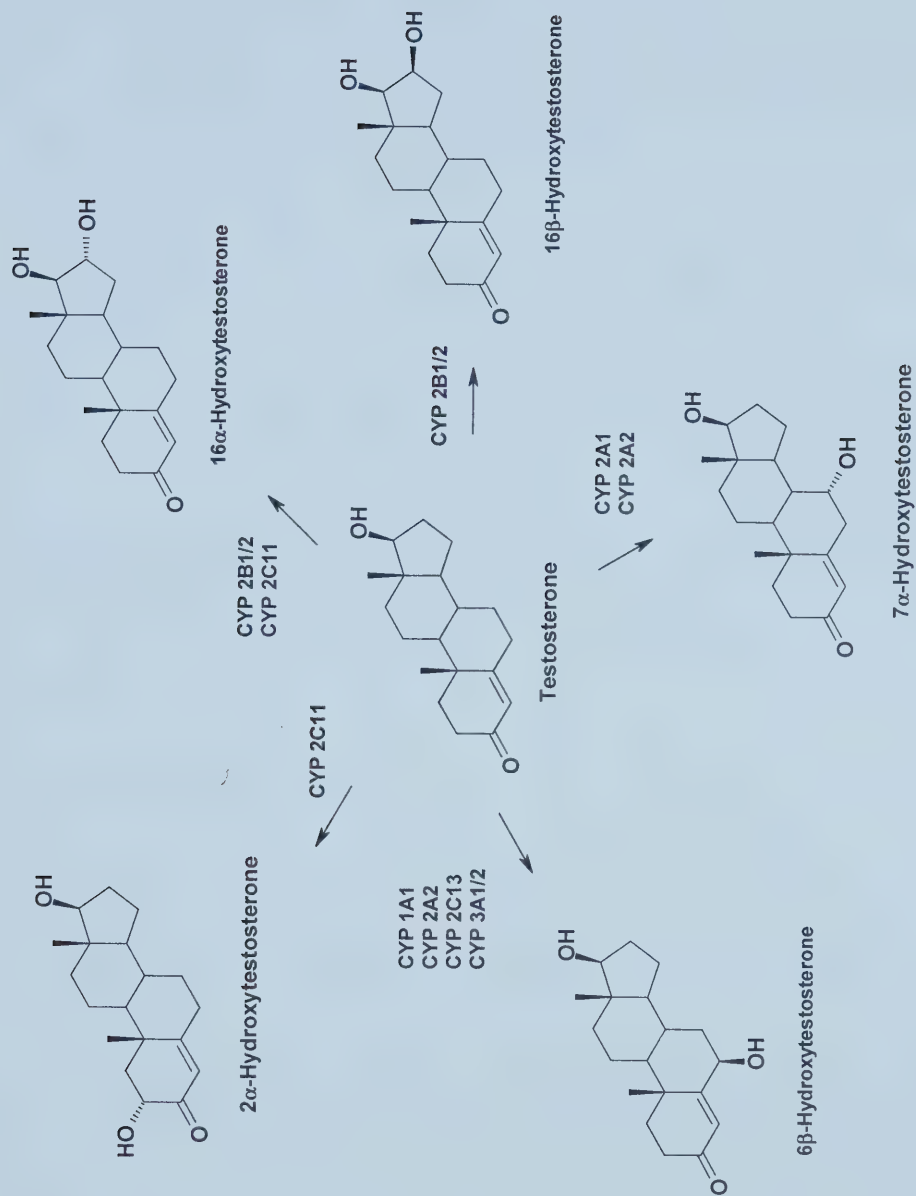


Fig 1.1 The major metabolic pathways of testosterone in rat



steroid nucleus provides a sensitive indicator for the identification of a specific CYP isozyme. A variety of chromatographic procedures have been developed for the analysis of hydroxylated metabolites of steroids including testosterone and progesterone (Wood et al 1983; van der Hoeven 1984; Sonderfan et al 1987; Swinney et al 1987; Waxman 1988; Arlotto et al 1991; Reinerink et al 1991; Waxman 1991; Sanwald et al 1995).

One approach to the simultaneous study of the activities of several CYPs is the determination of the regio- and stereo-selective hydroxylation of testosterone. Five pathways of testosterone hydroxylation (Figure 1.1) have been identified from incubations with microsomes using reversed-HPLC. They are associated with the activities of the following CYP isoforms: CYP 1A1/2, -2A1/2, -3A, -2B1, -2C (Waxman 1983, 1988; Wood 1983; Sonderfan 1987), which accounts for more than 80% of CYP enzymes.

## **1.6 Midazolam (MDZ)**

### **1.6.1 Chemical Properties of MDZ**

MDZ, 8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5- $\alpha$ ][1,4]benzodiazepine (Figure 1.2),  $C_{18}H_{13}ClFN_3$ , molecular weight 325.77, is an imidazole benzodiazepine. The imidazole ring is relatively basic ( $pK_a = 6.0$ ), thus allowing the preparation of salts, which are stable in water solution at  $pH < 4$ . At physiological pH the drug become much more lipid soluble (Gerecke 1983). Water solubility minimizes pain on injection and venous thrombosis compared with diazepam, which is administered in organic solvent.



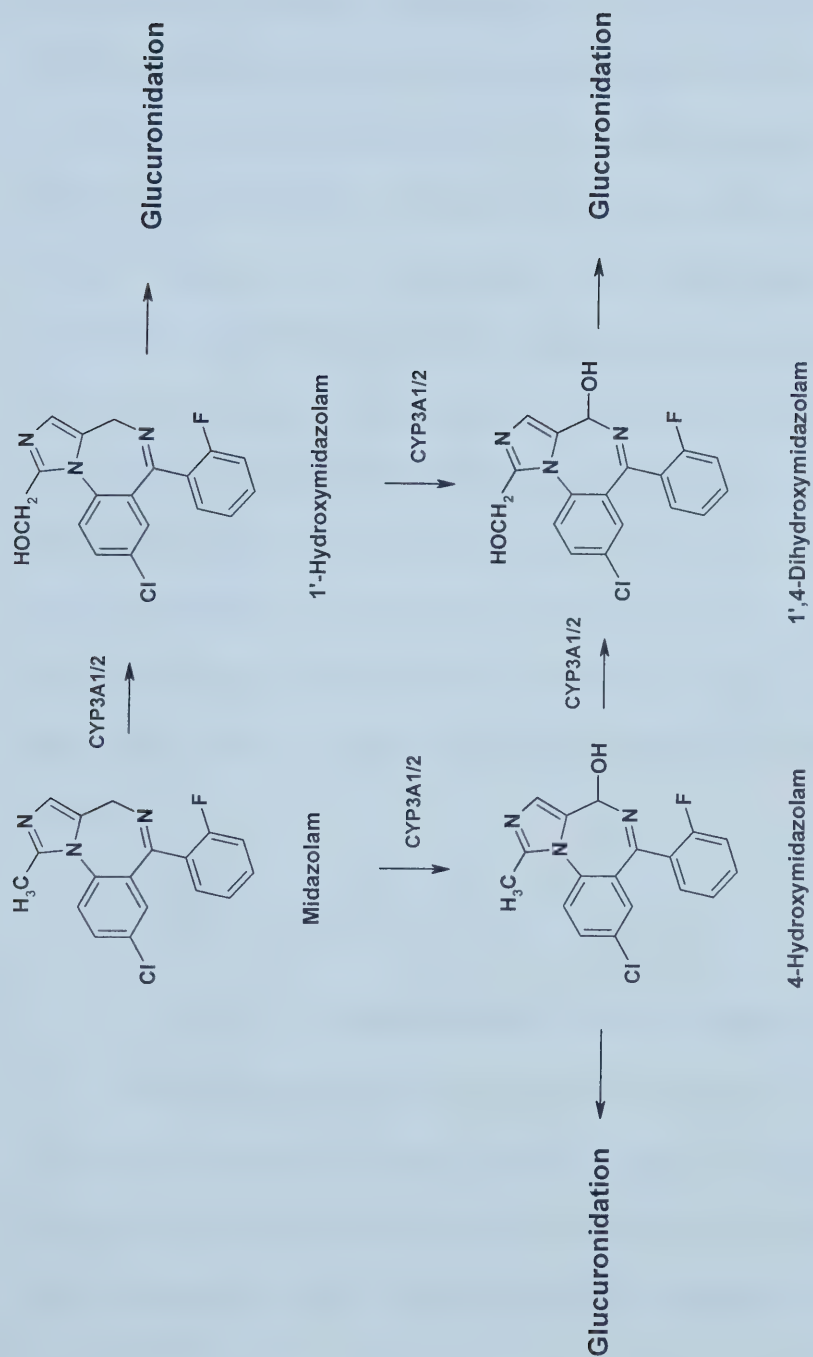


Figure 1.2 The metabolic pathways of midazolam in rat



### **1.6.2 Pharmacology of MDZ**

Although the water solubility of MDZ makes it different from many other benzodiazepines, the basic pharmacology of MDZ is similar to that of the other benzodiazepine derivatives such as diazepam in animal studies (Pieri et al 1981).

MDZ is a parenteral benzodiazepine with sedative, amnesic, anxiolytic, and muscle relaxant and anticonvulsant properties. The drug exerts its clinical effect by binding to a receptor complex, which facilitates the action of the inhibitory neurotransmitter  $\gamma$ -aminobutyric acid (GABA). MDZ has a faster onset and shorter duration of action than other benzodiazepines such as diazepam and lorazepam. It is water-soluble in the commercially prepared formulation but becomes lipid soluble at physiological pH and then crosses the blood brain barrier. It is metabolized in liver by the CYP system, and its principle metabolite is 1'-hydroxymethyl MDZ. The other two minor metabolites are 4-hydroxyMDZ and 1',4-dihydroxyMDZ. All three metabolites are conjugated to form glucuronide (Figure 1.2). Less than 1% of unchanged MDZ excreted primarily by the kidneys.

### **1.6.3 Animal Study**

#### **1.6.3.1 Pharmacokinetics of MDZ in Animals**

Although the pharmacokinetic profile of MDZ has been studied extensively in human, the information on animals, however, is quite limited. Several studies on the pharmacokinetics of different benzodiazepines in rats have been reported (Mandema et al 1991b; Mandema et al 1991a; Mandema et al 1992b; Mandema et al 1992a; Hoogerkamp



et al 1996; Lau et al 1996; Cleton et al 1998; Takedomi et al 1998; Tuk et al 1998; Yamano et al 1999).

In a chronic food-limited study, Lau et al. (1996) demonstrated the pharmacokinetic profiles of MDZ after four different administration routes (intravenous, subcutaneous, intraperitoneal, and oral). They found that the concentration versus time profile for all four routes of administration was best described by a bi-exponential equation. The rate of MDZ absorption was rapid, and the peak concentration ( $C_{\max}$ ) was attained in less than 7 minutes for the three extravascular routes. The total body clearance ( $CL_{\text{total}}$ ) was 2.03 l/h/kg and volume of distribution at steady-state ( $V_{\text{ss}}$ ) was 2.47 L/kg. Elimination half-life ( $T_{1/2\beta}$ ) of MDZ ranged from 23.1 to 49.5 minutes and metabolites could not be detected (Lau et al 1996). These kinetic parameters were consistent with those reported by others (Jack et al 1983; Sethy et al 1987).

In a series of studies carried out by Dandoff and colleagues (Cleton et al 1998; Tuk et al 1998; Cleton et al 1999), MDZ was given to rats as an intravenous infusion over 2 minutes at a dose of 10mg/kg. They found that  $T_{1/2\beta}$  were between 25 to 31 minutes,  $CL_{\text{total}}$  and  $V_{\text{ss}}$  were 6.6 l/h/kg and 2.5 l/kg, respectively.

Takedomi and colleagues studied the interaction of MDZ and histamine H<sub>2</sub>-receptor antagonists in rats. After a 10mg/kg infusion of MDZ, the average of  $T_{1/2\beta}$  and  $CL_{\text{total}}$  were 28.8 minutes and 4.2 L/hr/kg, respectively (Takedomi et al 1998). Similar results have been reported by Yamano with  $CL_{\text{total}}$  between 5.6 and 6.7 L/hr/kg and  $T_{1/2\beta}$  from 32.6 to 27.8 minutes after 10mg/kg infusion (Yamano et al 1999).



### **1.6.3.2 Metabolism of MDZ in Animals**

CYP enzymes are heme-containing proteins involved in the oxidative metabolism of thousands of compounds. At least 221 known genes encoding for CYP enzymes have been sequenced and divided into families based on peptide homology (Nelson et al 1993). CYP enzymes exist in species ranging from bacteria to man and are expressed throughout most tissues including liver, in which the highest amount of CYP protein is expressed. In addition to a very broad range of xenobiotics, CYP enzymes are also involved in the metabolism of the endogenous ligands such as steroids and fatty acids (Nelson et al 1993).

CYP3A2, originally isolated as a unique form in the liver of rats treated with pregnenolone-16 $\alpha$ -carbonitrile (PCN) (Elshourbagy & Guzelian 1980), has been shown to exist constitutively (Yanagimoto et al 1992). This form is highly inducible by glucocorticoids and has striking qualitative and quantitative difference between species in their responses (Wrighton et al 1985). The CYP3A2 protein is responsible for most of testosterone 6 $\beta$ -hydroxylase activity in rats (Gonzalez & Deis 1986).

The rat CYP3A subfamily consists of CYP isoforms 3A1 and 3A2, which are approximately 90% identical and functionally equivalent to human 3A3 and 3A4, respectively. CYP3A1 is difficult to detect in a normal untreated rat whereas CYP3A2 accounts for approximately 25% of total CYP in a rat liver (Imaoka et al 1988). CYP3A subfamily is involved with the 6 $\beta$ -hydroxylation of testosterone. Studies (Desjardins & Iversen 1995) showed that CYP3A2 protein concentration had a unique profile which paralleled that of steroid 6 $\beta$  hydroxylase activity.



The 3A family metabolizes numerous substrates including many clinically important drugs such as MDZ, nifedipine, erythromycin, tamoxifen and cyclosporine (Gonzalez 1989; Nelson et al 1993; Kumar et al 1994). CYPs 3A1 and 3A2 are both induced by macrolide antibiotics, PCN, phenobarbital and dexamethasone. MDZ metabolism is considered a specific *in vivo* pharmacological marker of CYP3A2 enzymatic activity (Watkins 1994; Higashikawa et al 1999).

#### **1.6.4 Human Study**

##### **1.6.4.1 Pharmacokinetics of MDZ**

The pharmacokinetic properties of MDZ have been determined in healthy volunteers (Heizmann et al 1983) and as a sedative in patients who are artificially ventilated in ICU (Pieri et al 1981; Malacrida et al 1992). After an intravenous injection, the MDZ plasma concentration versus time graph in human can generally be described by a bi-exponential equation (Armreim et al 1981). The  $T_{1/2\alpha}$  and  $T_{1/2\beta}$  are usually less than 30 minutes and between 1 and 3 hrs, respectively. After a single oral doses of MDZ 10 to 40mg,  $C_{\max}$  has been reported as early as 20 minutes and as late as 50 minutes, depending on sampling intervals (Allonen et al 1981; Smith et al 1981; Heizmann et al 1983; Sjovall et al 1983). There appears to be a linear relationship between oral dose and plasma concentration within the studied range (10 to 40 mg). Systemic availability after oral administration is 44% on average after a 15mg oral dose (Allonen et al 1981). The low bioavailability is apparently explained by first-pass hepatic extraction. The apparent volume of distribution fluctuates between 0.8 and 3 l/kg (Allonen et al 1981; Armreim et al 1981; Kienlen 1988). The kinetics of MDZ during continuous infusion was consistent with the single-



dose profile (Allonen et al 1981). MDZ is extensively bound to plasma proteins with only about 4% being unbound (Allonen et al 1981; Greenblatt et al 1984). The free fraction is higher in patients with chronic renal failure than that in those with normal renal function (Vinik et al 1983). Plasma clearance has been estimated to be 0.34 to 0.54 l/h/kg in healthy subjects (Allonen et al 1981; Smith et al 1981; Sjovall et al 1983; Greenblatt et al 1984). Plasma clearance decreased in elderly patients (Greenblatt et al 1984).

#### **1.6.4.2 Metabolism of MDZ in Human**

The CYP3A gene subfamily accounts for up to 30% of the total CYP presented in an adult human liver and for the majority of CYP in human intestine (Krishna & Klotz 1994; Shimada et al 1994). MDZ is eliminated rapidly and almost exclusively through CYP3A4-catalyzed by oxidation in human (Heizmann & Ziegler 1981). The main metabolic route of MDZ is *via* hydroxylation to form 1'-hydroxyMDZ. This major metabolite is the only pharmacologically active one, but its potency is markedly weaker than that of the parent drug. Clinically, this metabolite is of no significance as an active component, because immediately after its formation it is conjugated by glucuronic acid to form a pharmacologically inactive end product. In human, 60 to 70% of an orally administered dose of <sup>14</sup>C-MDZ was recovered in the urine as conjugated 1'-hydroxyMDZ (Heizmann & Ziegler 1981). The other two minor metabolites are 4-hydroxyMDZ and 1', 4-dihydroxyMDZ (Figure 1.2). The plasma concentrations of these two unconjugated metabolites are low. They are recovered in 3% and 1% respectively in urine. Furthermore, these two minor metabolites are also rapidly conjugated after their formation. (Vree et al 1981; Heizmann et al 1983; Jochemsen et al 1983).



#### **1.6.4.3 Factors Affecting MDZ Pharmacokinetics**

Since MDZ is eliminated by hepatic metabolism, one would expect liver disease would impair MDZ metabolism and hence its clearance (Trouvin et al 1988; Pentikainen et al 1989). The Trouvin group (Trouvin et al 1988) studied the pharmacokinetic profile of MDZ in cirrhosis patients. They found that after a 0.2mg/kg *iv* bolus of MDZ, the value of area under the curve (AUC) was up by 57% when compared to subjects in the control group;  $T_{1/2\beta}$  was prolonged 25% and  $CL_{total}$  was lowered by 37%. The authors claimed that the alterations in MDZ pharmacokinetics observed here might be explained by the moderate degree of liver dysfunction in patients with cirrhosis.

In healthy subjects, the protein bound fraction of MDZ is estimated to be 98% (Reves et al 1978) while lower (95%) in patients with cirrhosis (Trouvin et al 1988). Higher volume of distribution and plasma clearance are also found in patients with renal failure (Vinik et al 1983).

The pharmacokinetics of MDZ varies with age and gender. Children less than six months of age have a prolonged  $T_{1/2\beta}$  and decreased  $CL_{total}$  of MDZ than those in adults. This probably is a consequence of the time required for maturation of the hepatic microsomal oxidizing system over the first six months after birth (Kearns et al 1982). MDZ has a large volume of distribution of 0.96 to 6.6 L/kg in adults and tends to be higher in females and obese (Greenblatt et al 1984).  $V_{ss}$  is also higher in the elderly than in younger patients (Avram et al 1983).

#### **1.6.5 Rationale of Choosing MDZ as Model Drug**

In humans and in rats, the concentration versus time curves of MDZ could both be described by a bi-exponential equation although in rats shorter elimination half-life



and higher clearance have been reported when compared to human (Smith et al 1981; Pentikainen et al 1989). Studies showed that age altered the pharmacokinetic property of MDZ in humans (Greenblatt et al 1984) and in rats (Desjardins & Iversen 1995) in a parallel fashion.

The rat CYP3A subfamily consists of CYPs 3A1 and 3A2, which are approximately 90% identical and functionally equivalent to human 3A3 and 3A4, respectively. They also shared a numbers of common inducers such as dexamethasone, glucocorticoids (Yanagimoto et al 1992; Desjardins et al 1995; Nakajima et al 1999) and inhibitors such as ketoconazole (Wrighton & Ring 1994; Yamano et al 1999). MDZ is mainly metabolized by CYP3A4 in human and CYP3A2 in rat. (Imaoka et al 1988).

MDZ was almost exclusively metabolized by one isozyme both in humans and in rats to one major metabolite 1'-hydroxyMDZ, which is rapidly conjugated. The simplicity of its metabolic pathway makes it a suitable candidate to be used as a probe drug to study CYP3A enzymes. Therefore, we concluded that using MDZ as our probe drug to study the impact of PN on drug metabolism is appropriate.

## **1.7 Rationale for Choosing Sprague-Dawley (SD) Rat**

Rat is the most common animal model used to investigate PN-related hepatic abnormalities because of its small size, economic cost, availability, and ease of handling and maintenance. More importantly, data in literature prove that the effects of PN on drug metabolism appear to be similar between rats and humans. Therefore, the animal model chosen appears to be appropriate. CYP isoforms have been associated with gender



specificity. Such as CYP2C11 is expressed in male SD rats whereas CYP 2C13 only found in female rats. Due to this reason, only male Sprague-Dawley rats were used.

## 1.8 Hypotheses

Liver injuries induced by PN are caused by bacterial translocation, which is indicated by an increased level of endotoxin in portal system. The increase in endotoxin level is associated with the release of cytokines and nitric oxide. This sequence of events will lead to an alteration of drug elimination when the affected CYP isozymes are involved in the disposition of these drugs.

## 1.9 Objectives

1. To investigate the levels of endotoxin measured in the portal and peripheral circulation after PN treatment.
2. To measure levels of cytokines (TNF- $\alpha$ , IL-6 and IL-1 $\beta$ ) and nitrite in liver cytosol after PN treatment.
3. To examine PN effects on CYP isoforms (CYP 2C11, 2B1, 1A1, 3A2) activities *in vitro* using testosterone as a probe.
4. To study PN effects on drug pharmacokinetics *in vivo* using midazolam as a CYP 3A2 probe.



## **2 Materials and Experiments**

### **2.1 Animals**

Male Sprague-Dawley rats weighting 240-270 g were purchased from Biosciences Animal Service, University of Alberta. All animals were housed in the Dentistry-Pharmacy Building Animal Service and were fed conventional rodent chow (Richmond Standard, PMI Feed Inc., MO, US). The protocol of this study met the guideline of the Canadian Council on Animal Care and the use of animals was approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

### **2.2 Composition of PN**

The composition of the PN solution was identical to that reported by Zaman et al (Zaman et al 1997). Each liter of PN consists of 242 g dextrose (70% Dextrose Injection USP, Clintec Nutrition Company, ON, Canada), 52 g amino acids (10% Travasol<sup>®</sup> amino acid injection blend B with electrolytes, Clintec Nutrition Company, ON, Canada) (Table 2.1), 2 ml of multivitamins (Mulit-1000<sup>®</sup> multivitamin solution for IV infusion, SEBEX Inc., QC, Canada) (Table 2.2) and 2.25 mmol of calcium gulconate (Calcium Gluconate 10% Injection USP, Abbott Laboratories Limited, QC, Canada). Solutions were prepared aseptically in a laminar flow hood and containers were immediately covered with aluminum foil to avoid exposure to light.



**Table 2.1 Composition of Travasol 10% Blend B**

<u>Essential Amino Acid</u>	
L-Leucine	620 mg
L-Phenylalanine	620 mg
L-Methionine	580 mg
L-Lysine (HCl)	580 mg
L-Isoleucine	580 mg
L-Valine	460 mg
L-Histidine	440 mg
L-Threonine	420 mg
L-Typtophan	180 mg
<u>Non-Essential Amino Acid</u>	
L-Alanine	2.08 g
Aminoacetic Acid	2.08 g
L-Arginine	1.04 g
L-Proline	420 mg
L-Tyrosine	40 mg
<u>Electrolyte (milliequivalents/liter)</u>	
Sodium	70
Potassium	60
Magnesium	10
Acetate	150
Chloride	70
Phosphate (as $\text{HPO}_4^{=}$ )	60



**Table 2.2 Composition of M.V.I.-12 multivitamin solutions in each 5 ml vial**

Ascorbic acid (Vitamin C)	100 mg
Vitamin A (retinal)	1 mg
Ergocalciferol (Vitamin D)	5 mcg
Thiamine (Vitamin B <sub>1</sub> ) as the hydrochloride	3 mg
Riboflavin (Vitamin B <sub>2</sub> ) as riboflavin-5-phosphate sodium	3.6 mg
Pyridoxine HCl (Vitamin B <sub>6</sub> )	4 mg
Niacinamide	40 mg
Dexpantenol (d-pantothenyl alcohol)	15 mg
Vitamin E (dl-alpha tocopheryl acetate)	10 mg (c)
Biotin	60 mcg
Folic acid	400 mcg
Cyanocobalamin (Vitamin B <sub>12</sub> )	5 mcg



## 2.3 Experimental Protocols

There were two major protocols in the current study: an *in vitro* study and an *in vivo* study. The purpose of the *in vitro* study was to measure PN effects on the release of endotoxin, cytokines, free radicals and CYP enzyme activities. The *in vivo* study was designed to explore the impact of PN on pharmacokinetics of MDZ, a model drug to evaluate CYP3A activity for rats and humans.

### 2.3.1 *In vitro* Study

Fourteen male Sprague-Dawley rats weighing 240-270 g went through jugular vein (JV) cannulation under light isoflurane anesthesia. After 72 hr recovery from surgery, they were randomly assigned to one of two groups: a) PN-treated group (PN, n=7), receiving continuous infusion of the PN solution at a rate of 3 ml/hr or b) control group (control, n = 7), receiving intravenous saline infusion at the same rate while having free access to normal rodent chow. Both groups had access to water *ad libitum*. All infusions were delivered by a volumetric infusion pump (model 927, IMED Corporation, CA, US) to the JV catheters. Treatments lasted for seven continuous days. On the eighth day, a 2.5 ml of blood from both portal vein (PV) and inferior vena cava (IVC) were collected into pyrogen-free vacutainer tubes (SST<sup>®</sup> Gel and Clot Activator vacutainer, Becton Dickinson Corporation, NJ, US). The blood samples were allowed to sit at 4<sup>0</sup> C for at least one hour before serum was harvested. Then the serum was transferred into endotoxin-free polystyrene tubes (Fisher Scientific Corp., ON, Canada). The liver was perfused and microsome was prepared (See section 2.10.2.2. for detailed procedures). All samples were stored at -80 °C until analysis. The level of endotoxin was measured using



a LAL kit (LAL, Kinetic QLC-1000 test kit, BioWhittaker, MD, US). Cytokines TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were measured using commercially available ELISA kits (Cytoscreen<sup>TM</sup> immunoassay, BioSource, CA, US). Nitrite, the end product of NO, was measured using Griess Reagent (Arias-Diaz 1995). Protein content in microsomes was determined using the standard Bradford method (Bradford 1976) and the total CYP enzyme activity was measured using a carbon monoxide binding method (Omura & Sato 1964). With specific antibodies, Western-blotting was performed to qualitatively evaluate changes of individual CYP isozymes. In order to study the activities of individual CYP isozymes quantitatively, microsomes were incubated with testosterone (Stereloids Inc., NH, US); concentrations employed ranged from 10  $\mu$ M to 750  $\mu$ M. The formations of its five metabolites (6 $\beta$ , 7 $\alpha$ , 16 $\alpha$ , 16 $\beta$ , 2 $\alpha$ ) were quantified using HPLC and  $V_{max}$  and  $K_m$  values were estimated using SigmaPlot (SPSS, Incorporation, CA, US).

### **2.3.2 *In vivo* Study**

Twelve rats were subject to both carotid artery (CA) and JV cannulations. After recovery from surgery, they were divided into two groups and received the same treatments as described in the *in vitro* study. On the eighth day, the rats were weighed and given a 3 mg/kg *iv* bolus dose of MDZ through JV catheter. The solvent was a mixture of 20% ethanol, 20% PEG400 and 60% water and the concentration was 3 mg/ml. A 250  $\mu$ l of blood sample was taken from CA at 0, 2.5, 5, 7.5, 10, 15, 30, 45, 60, 90, 120, and 180 minutes after dosing. The blood samples were collected in pre-heparinized plastic tubes (Becton Dickson Corporation, NJ, US) and immediately centrifuged at 1000 *g* (Fisher Scientific Incorporate, ON, Canada) for 15 minutes to obtain plasma. The plasma was then transferred into a clean tube (VWR CANLAB, AB, Canada) and stored at -20 $^{\circ}$  C



until analysis. The plasma concentrations of MDZ were measured using HPLC. Pharmacokinetic parameters were calculated using WinNonlin (Pharsight Corporation, NC, US).

## **2.4 Surgery**

### **2.4.1 JV Cannulation**

A catheter was prepared using a 3.5 cm long silastic tubing (o.d. 0.047" and i.d. 0.025", Dow Corning Corporation, MI, US), which was attached to one end of a 40 cm long PE-50 tubing (I.D. 0.023" and O.D. 0.038", Becton Dickinson Primary Care Diagnostics, MD, US). The other end of the PE-50 tubing was connected to a 23 *gauge* needle fitted to a 1 ml syringe filled with heparinized saline (20 unit/ml). The void volume of the catheter was then filled with heparinized saline to ensure no air bubbles were present. The animal was anesthetized using isoflurane (Bimeda-MTC Animal Health Inc., ON, Canada). When the rat was fully unconscious, which was confirmed by the loss of toe pinch (pedal) reflex, it was shaved in the middle area between two jaws and the nape area. The shaved areas were scrubbed with isopropyl alcohol and the rat was placed on its back. The rat was closely monitored during the surgery. An incision (1.5 – 2.0 cm) in the middle of the left and right clavicles was made and the right JV was exposed using blunt dissection. Two pieces of 4-0 silk (Fine Science Tools Incorporation, BC, Canada) were threaded under both the anterior and posterior side of the JV. The anterior side of the vein was occluded with the thread. A micro vascular clamp (Fine Science Tools Incorporation, BC, Canada) was applied to the posterior end. A small incision was made in the exposed vein between the two ligatures. Using a pair of



Dumont forceps (Fine Science Tools Incorporation, BC, Canada), the catheter was introduced into the JV. Subsequently, the vascular clamps were released and the catheter was advanced approximately 3.0 cm into the vein. The proper position of the catheter was ascertained by withdrawing blood from the catheter. Once in place, the catheter was flushed with 0.2 ml of the heparinized saline (20 unit/ml) (Heparin Sodium Injection B.P., Leo Pharma Incorporation, ON, Canada) and secured by ligating the two threads. Then the catheter was tunneled subcutaneously and exteriorized through a very small incision in the nape where it was connected to a coiled metallic spring mounted on a swivel (Rodent Single Channel Swivel, Harvard Apparatus, QC, Canada). This connection permitted free mobility of rats. The the incisions were closed with a 4-0 silk suture. Finally, a 0.09 ml of heparinized saline (500 unit/ml) was introduced into the catheter as a heparin lock to prevent clotting during the recovery period. The rat was housed individually in a metabolic cage with free access to food and water. The housing facility was maintained with a 12-hr light and dark cycle. The animal was allowed to recover for 72 hrs before initiation of a study. The catheter was flushed with 20 unit/ml of heparin and the heparin lock was replaced daily until the infusion started.

#### **2.4.2 CA Cannulation**

The catheter for CA cannulation was prepared exactly the same way as that for JV except 1.0 cm long silastic tubing was used as a support and was attached at 2.5 cm posterior to the PE50 tube. The surgical procedure was also the same as that described for JV cannulation except only 2.5 cm of catheter was introduced into the CA. The catheter was flushed with 20 unit/ml heparin daily until the end of study.



## **2.5 Endotoxin Measurement**

Endotoxin level was measured using a pyrogen test kit called LAL Kinetic-QCL 1000 (BioWhittaker Inc., MD, US). It was a kinetic assay for quantifying Gram-negative bacterial endotoxin. In brief, a 50 µl sample was simply mixed with the LAL/SUBSTRATE reagent, and then placed in the Kinetic-QCL reader that automatically monitored over time for the appearance of a yellow color. The time required for the appearance of the yellow color (Reaction Time) was inversely proportional to the amount of endotoxin presented in the sample. In other words, reaction occurred rapidly in the presence of a large amount of endotoxin. The concentration of endotoxin in unknown samples was calculated using a standard curve.

A 100 µl of blank, endotoxin standards, samples, and positive controls were each carefully dispensed into appropriate wells of a microplate (VWR CANLAB, AB, Canada). The plate was incubated in a 37<sup>0</sup> C incubation chamber (Anthos HT3 ELISA Reader, Anthos Labtec Instruments, Salzburg, Austria) for 10 minutes. During this time, an appropriate volume of Kinetic-QCL reagent was reconstituted gently with LAL Reagent Water according to the manufacturer's instructions. After 10 minutes of incubation, a 100 µl of the reconstituted reagent was added carefully into each well to avoid bubble generation. Then the reader was activated to monitor the absorbance at 405 nm (Anthos HT3 ELISA Reader, Anthos Labtec Instruments, Salzburg, Austria).

## **2.6 Cytokine Measurement**

ELISA kits were purchased from BioSource International, Inc. (Cytoscreen<sup>TM</sup> immunoassay Kit, BioSource International, Inc., CA, US).



### 2.6.1 IL-6

In order to measure the concentrations of IL-6 in liver cytosol samples, a 96-well plate (cell wells<sup>TM</sup> 96 well plate, Corning Laboratories, CA, US) was used to perform the ELISA test and all standards and samples were run in duplicate. For those wells used to generate standard curves, a 100 µl of pre-diluted standards (provided with the kit) with concentrations ranging from 31.2 to 2000 pg/ml was added to appropriate wells. For those wells reserved for samples, a 50 µl of Standard Diluent Buffer (provided with the kit) was first added to the appropriate wells followed by the addition of a 50 µl of samples. The wells used as background were left empty. The plate was tapped gently on the side to promote mixing. Then the plate was covered and incubated for 2 hrs at 37<sup>0</sup> C. At the end of incubation, a 100 µl of biotinylated anti-IL-6 (Biotin Conjugate, provided with the kit) was pipette into each well except for the background ones. The plate was incubated again for one hour at room temperature and washed thoroughly with the washing buffer (provided by the kit). After that, a 100 µl of pre-prepared Streptavidin-HRP solution (provided with the kit) was added to each well except for the background ones and the plate was incubated for another 30 minutes at room temperature. After another thorough washing with the washing buffer, a 100 µl of Stabilized Chromogen (provided with the kit) was added to every well including the background ones. The plate was then left in dark at room temperature for 30 minutes for color development. Finally, after adding a 100 µl of stop solution (provided with the kit) to each well, the absorbance of each sample was measured at 450nm using a microplate reader (Anthos HT3 ELISA Reader, Anthos Labtec Instruments, Salzburg, Austria).



The standard curve had IL-6 concentrations ranging from 31.2 to 2000 pg/ml. Within this range, the correlation coefficient values of standard curves were higher than 0.98. The lowest quantifiable concentration was 8 pg/ml. Both intra-day and inter-day assay precisions were less than 10%.

### **2.6.2 TNF- $\alpha$**

The concentration of TNF- $\alpha$  in liver cytosol samples was measured by a commercial available ELISA kit (Cytoscreen<sup>TM</sup> immunoassay Kit, BioSource International, Inc., CA, US) using a 96-well plate (Cell Wells<sup>TM</sup> 96 well plate, Corning Laboratories, CA, US ). All the following procedures were performed at room temperature. All standards and samples were run in duplicates. A 50  $\mu$ l volume of pre-diluted standards (provided with the kit) or samples was added to an appropriate well followed by an addition of a 50  $\mu$ l of biotinylated anti-TNF- $\alpha$  (provided with the kit). The wells for background measurement were left empty. The prepared plate was first tapped gently on the side for mixing, covered and incubated for 1.5 hrs. After a thorough washing with the washing buffer (provided with the kit), a 100  $\mu$ l Streptavidin-HRP Working solution (provided with the kit) was added into each well except the background ones. The plate was covered and incubated again for 45 minutes. After another thorough washing with the washing buffer, 100  $\mu$ l of Stabilized Chromogen (provided with the kit) was added into every well including the background ones and the plate was left in the dark for 30 minutes color development. Finally, after adding 100  $\mu$ l of the stop solution (provided with the kit), the absorbance was measured at 450 nm using a microplate reader (Anthos HT3 ELISA Reader, Anthos Labtec Instruments, Salzburg, Austria).



The standard curve has concentrations ranging from 15.6 to 1000 pg/ml. The correlation coefficient values of standard curves were higher than 0.98. The lowest quantifiable limit was 4 pg/ml. Both intra-day and inter-day assay precisions were less than 10%.

### **2.6.3 IL-1 $\beta$**

In order to measure the concentrations of IL-1 $\beta$  in liver cytosol samples, a 96-well plate (cell wells<sup>TM</sup> 96 well plate, Corning Laboratories, CA, US ) was used to perform the ELISA test (Cytoscreen<sup>TM</sup> immunoassay Kit, BioSource International, Inc., CA, US). The following procedures were performed at room temperature. All standards and samples were run in duplicate. A 100  $\mu$ l volume of a pre-diluted standard (provided with the kit) ranging from 31.2 to 2000 pg/ml was added to an appropriate well. For wells reserved for samples, a 50  $\mu$ l of Standard Diluent Buffer (provided with the kit) was first added to each well followed by a 50  $\mu$ l of sample. The wells reserved for background were left empty. The plate was tapped gently at the side, covered and incubated for 3 hrs. After a thorough washing with the washing buffer (provided by the kit) at the end of incubation, a 100  $\mu$ l of biotinylated anti-IL-1 $\beta$  (Biotin Conjugate, provided with the kit) was pipette into each well except for the background ones and the plate was incubated for another hour. After a second wash with the washing buffer, a 100  $\mu$ l Streptavidin-HRP Working solution (provided with the kit) was added to each well except the background ones and the plate was again incubated for 30 minutes. After a third wash, a 100  $\mu$ l of Stabilized Chromogen (provided with the kit) was added into every well including the background ones and the plate was left in the dark for a 30 minute color development.



Finally, a 100 µl volume of stop solution (provided with the kit) was added into every well and the absorbencies were measured at 450nm using a microplate reader (Anthos HT3 ELISA Reader, Anthos Labtec Instruments, Salzburg, Austria).

The standard curve has concentrations ranging from 31.2 to 2000 pg/ml. The correlation coefficient values of standard curves were higher than 0.98. The lowest quantifiable limit was 3 pg/ml. Both intra-day and inter-day precisions were less than 10%.

## **2.7 Nitrite Measurement**

NO was determined as nitrite ( $\text{NO}_2^-$ ) concentration in the cytosol samples using Griess Reagent (Arias-Diaz et al 1996). One hundred microgram of naphthalenediamine (N-(1-naphthyl)ethylenediamine) dihydrochloride (MW 259.2) (Aldrich, WI, US) was dissolved in 100 ml double distilled water as “Solution A”. One g of sulfanilamide (MW 172.21) (Aldrich, WI, US) was dissolved in 5.88 ml of 85% phosphoric acid and 94.12 ml of double distilled water as “Solution B”. Solutions A and B were stable for a few weeks if stored at 4<sup>0</sup> C. The Griess Reagent was prepared freshly on the day of the experiment by mixing solutions A and B in a ratio of 1:1 and this reagent was only stable for several hours. Sodium nitrite (MW 69; 8.63 mg) (Aldrich, WI, US) was dissolved in an appropriate amount of double distilled water to make a 50 mM stock solution and stored at 4<sup>0</sup> C.

The standard curve was prepared by diluting the stock solution into at least 5 different concentrations ranging from 62.5 to 375 nM. A 100 µl volume of each standard and sample was pipetted into an appropriate well in triplicates. A 100µl volume of freshly prepared Griess Reagent was added into each well. The optical density of the microplate



was read on a microplate reader (Anthos HT3 ELISA Reader, Anthos Labtec Instruments, Salzburg, Austria) at 560nm immediately after formation of the color. The concentrations of nitrite in samples were calculated according to the calibration curve.

## **2.8 Western-Blot Study**

### **2.8.1 Chemicals**

All equipments and materials for conducting Western Blot and chemicals for preparing separating gel, stacking gel and sample buffer were purchased from Bio-Rad Laboratories, CA, US. The other chemicals were purchased from Sigma, MO, US. Antibodies were purchased from Genetest Corporation, MA, US. These antibodies were imported from Daiichi Pure Chemical, Japan.

### **2.8.2 Buffer Preparation**

To prepare the separating gel buffer, 7.5 ml double distilled water, 3.75 ml 4xTris/SDS pH8.8, 3.75 ml 40% acrylamide, 80  $\mu$ l freshly prepared 10% APS, and 8  $\mu$ l TEMED were mixed in a plastic test tube and labeled as Buffer A.

To prepare the stacking gel buffer, 3.75 ml double distilled water, 1.25 ml 4xTris/SDS pH6.8, 0.5 ml 40% acrylamide, 30  $\mu$ l freshly prepared 10% APS, and 5  $\mu$ l TEMED were mixed and labeled as Buffer B.

Both Buffer A and B were prepared freshly and used right after the mixture to form gels.

To prepare concentrated running buffer (x 5, pH 8.3), 9 g Tris base, 43.2 g glycine and 3 g SDS were dissolved into 600 ml of double distilled water and the solution was



stored at 4<sup>0</sup> C. For each run, 60 ml of this concentrated stock solution was diluted with 240 ml of double distilled water.

To prepare transfer buffer, 3.03 g Tris and 14.4 g glycine were dissolved into 800 ml of double distilled water, then 200 ml of methanol was added to the mixture to give 25 mM Tris, 192 mM glycine and 20% v/v methanol with a pH value of 8.3. The buffer was stored at 4<sup>0</sup> C.

To make concentrated PBS (x 10), 80 g NaCl, 14.4 g Na<sub>2</sub>HPO<sub>4</sub>, 2 g KCl and 2.4 g KH<sub>2</sub>PO<sub>4</sub> were dissolved into 950 ml of double distilled water. The buffer was adjusted pH to 7.4 using HCl and added to 1000ml. The solution was stored at room temperature.

To prepare sample buffer, 3.8 ml double distilled water, 1.0 ml 0.5M Tris-HCl, pH6.8, 0.8ml glycerol, 1.6 ml 10%(w/v) SDS, 0.4 ml 2-mercaptoethanol, and 0.4 ml 1%(w/v) bromophenol blue were mixed and the buffer was stored at room temperature.

### **2.8.3 Procedures**

A Western Blot study has three major steps: separating, transferring and immunoblotting. CYP enzymes in microsomal samples were first separated by electrophoresis on polyacrylamide gel (separation) and then the samples were electrophoretically blotted onto nitrocellulose membranes (transferring). Finally, expression levels of CYP enzymes in microsomal samples were qualitatively measured by immunoblotting polyclonal antibodies to individual CYP isoforms (immunoblotting).

#### **2.8.3.1 Separating Procedure**

Discontinuous polyacrylamide gel cassette consisted of separating (lower) gels and stacking (upper) gels. Buffer A (the separating gel buffer) was first poured into the



pre-assembled gel cassette and left for 45 to 60 minutes to polymerize and form the gel (the separating gel). Then a comb was placed on the top of the gel cassette to save the space for the sample loading wells before Buffer B (the stacking gel buffer) was poured into the gel cassette on the top of the separating gel. It took another 30 to 45 minutes to polymerize and form the stacking gel before the comb was finally pulled out slowly and gently. The gel cassette was then ready for the sample loading. Before loaded into the wells, the samples (10  $\mu$ g) were first diluted with sample buffer in a ratio of 1:6 and heated in boiling water for five minutes. After the samples were loaded, the gel cassette was placed into the running tank filled with running buffer. The running tank was connected to two electrodes. The electronic condition was set as 100 volt/30mA and the run was conducted for about one hour. At the end, the gel with separated protein on it was carefully removed from the gel cassette and rinsed in transfer buffer several times.

#### **2.8.3.2 Transfer Procedure**

Before the transfer procedure started, the transfer cassette was pre-assembled according to the manufacturer's instructions. The assembled cassette was inserted into the tank, which was filled with transfer buffer. An ice pad was put beside the cassette to absorb heat, which was generated during the run. Then the tank was covered and connected to the proper electrodes. The electronic condition was set at 100volt/200mA and the duration of the run was about one hour. At the end, the membrane was taken out from the cassette carefully and rinsed in PBS, then left in dark to dry.



### **2.8.3.3 Immunoblotting Procedure**

The membrane was first soaked in PBS with 5% dry milk for one hour, followed by soaking in diluted primary antibody (BioRad Laboratories, NY, US) for another hour (The primary antibody was pre-diluted in PBS with 0.5% dry milk based on the manufacturer's instructions). Then the membrane was washed in PBS with 0.1% Tween 20 for 5 minutes and the wash procedure was repeated three times. After that, the membrane was soaked again for one hour in secondary antibody (Sigma, MO, US), which was pre-diluted in PBS with 0.5% dry milk. After another three times washing, the membrane was finally soaked in staining solution (BioRad Laboratories, NY, US) for 2 to 3 minutes till the desired color developed. The membrane was then taken to a photo service for photography.

## **2.9 Histological Examination**

After perfusion with 1.15% ice-cold KCL, a small portion of liver was cut and stored in 10% formalin prior to being stained with hematoxylin-eosin, trichrom stain. Histology examination was done in a blinded fashion in the Department of Laboratory Medicine and Pathology, University of Alberta. The number of Kupffer cells in three separate linear area of 0.475 mm was counted.

## **2.10 *In vitro* study**

### **2.10.1 Chemicals**

Testosterone (4-androsten-17 $\beta$ -ol-3-one), and its five rat metabolites 6 $\beta$ -hydroxytestosterone (4-androsten-6 $\beta$ ,17 $\beta$ -diol-3-one), 7 $\alpha$ -hydroxytestosterone (4-



androst-7 $\alpha$ ,17 $\beta$ -diol-3-one), 16 $\alpha$ -hydroxytestosterone (4-androst-16 $\alpha$ ,17 $\beta$ -diol-3-one), 16 $\beta$ -hydroxytestosterone (4-androst-16 $\beta$ ,17 $\beta$ -diol-3-one), and 2 $\alpha$ -hydroxytestosterone (4-androst-2 $\alpha$ ,17 $\beta$ -diol-3-one) were purchased from Steraloids Incorporation, NH, US. The internal standard cortexolone was purchased from Aldrich Chemical Corporation WI, US. All other chemicals were purchased from Sigma, MO, US or Fisher Scientific Incorporate, ON, Canada unless stated otherwise. And all these reagents were analytical grade and solvents were HPLC grade.

## **2.10.2 Preparation of Liver Microsomes**

### **2.10.2.1 Buffer Preparation**

A 500 ml volume of 100 mM  $\text{KH}_2\text{PO}_4$  (MW 136.09) and 500 ml of 100 mM  $\text{K}_2\text{HPO}_4$  (MW 174.18) (analytical reagent, BDH Inc., CA, US) were prepared separately in two glass flasks. Using a pH meter (Accumet<sup>®</sup> pH meter 915, Fisher Scientific Incorporate, US), the acidic  $\text{KH}_2\text{PO}_4$  solution was gradually added to the basic  $\text{K}_2\text{HPO}_4$  solution to adjust the pH to 7.4. The buffer was labeled as Buffer A and stored at 4<sup>0</sup> C.

Another 100 ml of each above potassium phosphate salt solutions at a concentration of 100 mM were prepared. EDTA was weighed and slowly poured into the  $\text{K}_2\text{HPO}_4$  solution to give final concentration of 1 mM. Then the pH was measured and adjusted to 7.4 by adding the  $\text{KH}_2\text{PO}_4$  solution. Finally, 20ml glycerol was poured into a graduated cylinder and diluted to 100 ml using the pH 7.4 solution. The buffer was labeled as Buffer B and stored at 4<sup>0</sup> C.



### 2.10.2.2 Procedures

A liver microsome sample was prepared according to the standard operation procedure (SOP) in the laboratory. Rats (body weight 240-270 g) were lightly anesthetized under isoflurane and the abdominal cavity was opened for PV cannulation. A 16 gauge Quik-Cath<sup>®</sup> catheter (Baxter Health Care Corporation, IL, US) was inserted into the PV and then the liver was perfused *in situ* with ice-cold 1.15% KCl solution for 2 minutes at a flow rate of 30 ml/min to remove blood. The liver was then excised with minimal vascular, gut, and connective tissue attached. The following steps were performed either on ice or in a 4<sup>0</sup>C cold room. The liver was finely minced and homogenized using a glass homogenizer equipped with a glass pestle (Glas-Col<sup>®</sup>, Cole-Parmer Instrument Co., IN, US). The homogenates were transferred into plastic centrifuge tubes (Beckman Instruments Inc., CA, US) and centrifuged at 10,000 x g for 30 minutes using a Model IEC B020A centrifuge equipped with a No. 870 rotor (International Equipment Company, MA, US). The supernatant was carefully and evenly transferred into a polycarbonate ultracentrifuge tube (Ultratube<sup>®</sup>, 13 x 64 mm, Nalge Company, NY, US) and centrifuged at 100,000 x g for 60 minutes in a Model L8-55 ultracentrifuge (rotor type 503 Ti, Beckman<sup>®</sup> Instruments Inc., CA, US). The upper layer cytosol was then transferred into a clean tube and stored at -80<sup>0</sup> C for future cytokine measurement. The remaining microsome pellet was washed and resuspended in 100mM potassium phosphate buffer (pH 7.4, Buffer A). After re-centrifugation at 100,000 x g for another 60 minutes, the final microsome pellet was resuspended in 100 mM potassium phosphate buffer containing 1mM EDTA and 20% v/v glycerol (Buffer B) and stored at -80<sup>0</sup> C for maximum six months.



### **2.10.3 Determination of Microsomal Protein Content**

Microsomal total protein content was quantified using a commercial protein assay kit (BioRad Protein Assay, BioRad Laboratories, NY, US). The lyophilized bovine serum albumin standard provided with the kit was reconstituted with an appropriate volume of water to obtain a stock solution. The stock solution was then diluted into five different concentrations ranging from 8.0 to 80 mg/ml for the production of a standard curve. To determine the protein concentration in liver microsomal samples, the original sample was first diluted by a factor of 25 to 1000. A 160 µl of standard or diluted sample was pipetted in triplicates into appropriate wells in a 96-well plate (Cell Wells™ 96 well plate, Corning Laboratories, CA, US), followed by adding a 40 µl of the dye reagent (provided with the kit) to each well. The contents of each well were mixed thoroughly and the plate was left at room temperature for 10 minutes. Then the absorbance at 595 nm was monitored using a microplate reader (Anthos HT3 ELISA Reader, Anthos Labtec Instruments, Salzburg, Austria).

### **2.10.4 Determination of Total CYP Activities**

Total CYP enzyme activities were determined using carbon monoxide binding method (Omura & Sato 1964). The concentrated microsomal suspension was diluted into 6 ml of Buffer A (see “microsome preparation” section) to give a final concentration of 1.5 – 2.0 mg/ml. The diluted microsomal sample was equally distributed into two cuvettes and labeled as R for reference and S for sample. A few milligrams of sodium dithionite (sodium hydrosulfite,  $\text{Na}_2\text{S}_2\text{O}_4$ ) were added to both R and S cuvettes. Two minutes later, carbon monoxide (CO) was gently bubbled into S cuvette for one minute.



After another five minutes, the absorbance at 450 nm for S cuvette was recorded using R cuvette as blank.

### **2.10.5 Incubation Procedure**

#### **2.10.5.1 Standard Solution**

Stock solutions for the microsome incubation study were prepared as follow: Solutions of  $\text{MgCl}_2$  and  $\text{MnCl}_2$  and phosphate buffer were prepared in double distilled water to a final concentration of 250 mM, 250  $\mu\text{M}$  and 100 mM, respectively. Solutions of reduced  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADPH) were prepared freshly on the day of the experiment by dissolving sufficient NADPH in the phosphate buffer to give a final concentration of 10 mM. A mixture of the five testosterone metabolite standards was prepared in methanol. The final concentration of each was 50  $\mu\text{M}$ . Substrate testosterone and internal standard cortexolone were weighed and dissolved in methanol to a final concentration of 2.5 mM and 10 nM, respectively. All stock solutions were stored at  $-20^\circ\text{C}$  and used within three months.

#### **2.10.5.2 Procedure**

A microsomal reaction mixture (final volume, 500  $\mu\text{l}$ ) containing microsomal protein (0.5 mg), 10  $\mu\text{l}$  of  $\text{MgCl}_2$  solution (250 mM), 10  $\mu\text{l}$  of  $\text{MnCl}_2$  solution (250  $\mu\text{M}$ ), 50  $\mu\text{l}$  of fresh-prepared NADPH solution (10 mM) and 380  $\mu\text{l}$  of phosphate buffer (100 mM) (Buffer A, see “preparation of liver microsomes” section) was pre-incubated in a water bath at  $37^\circ\text{C}$  for 5 minutes. The metabolic reaction was initiated by adding 50  $\mu\text{l}$  of pre-warmed testosterone solution into the mixture while vortexing well. This mixture was



incubated for another 15 minutes. At the end of incubation, the mixture was transferred into a glass test tube containing 6 ml of dichloromethane ( $\text{CH}_2\text{Cl}_2$ ) to quench the reaction. The substrate concentrations for incubation mixture ranged from 10 – 750  $\mu\text{M}$ . Based on preliminary data, the formation of each metabolite studied reached a plateau within this range and the curves could be best fitted using Michaelis-Menten equation.

### **2.10.6 HPLC Assay**

#### **2.10.6.1 Instrumentation and Chromatographic Conditions**

A HP 1100 series HPLC system was used. Chromatographic separation of testosterone and its five metabolites was achieved a reverse-phase Eclipse XDB-C8 4.6mm ID X 7.5 cm, 3.5  $\mu$  column (Phenomenex Corporation, CA, US). An isocratic elution method was used for the quantification of testosterone and its metabolites in liver microsome samples. The mobile phase consisted of 43% methanol and 57% water. The flow rate was 1.0 ml/min and the temperature was set at 40<sup>0</sup>C. The UV signal was monitored at 247 nm. The retention times for internal standard, testosterone and its 6 $\beta$ -, 7 $\alpha$ -, 16 $\alpha$ -, 16 $\beta$ -, and 2 $\alpha$ -hydroxylated metabolites were 12.9, 26.3, 4.1, 4.5, 6.5, 8.3, 10.3 minutes respectively.

#### **2.10.6.2 Sample Preparation and Extraction**

For quantitative measurement of testosterone metabolites after incubating liver microsome with testosterone, a 500  $\mu\text{l}$  portion of incubated microsome mixture was transferred into a glass tube (16 x 100 mm, Kimax<sup>®</sup>, IL, US) containing 6 ml of  $\text{CH}_2\text{Cl}_2$  to stop the reaction. Then a 100  $\mu\text{l}$  of internal standard (cortexolone, 10 nM) was added



into each sample. The tube content was mixed vigorously for 10 minutes and centrifuged at 1000 g for another 10 minutes. The upper layer was discarded and the organic extract was dried under a gentle stream of nitrogen. Finally the dried test tube content was reconstituted with 130 µl of 20% methanol and then 50 µl of it was injected into HPLC system.

#### **2.10.6.3 Data Analysis**

Calibration curves were used to quantify testosterone metabolites. The concentration range for the standards was from 1 µM to 40 µM. This was achieved by serially diluting the stock solution into 5 different concentrations. The standards were spiked into blank microsome and extracted exactly in the same manner as those for samples. The standard curves were constructed by plotting the peak area ratios of metabolites to the internal standard against that of the spiked metabolites. The calibration curves were all linear ( $r^2 > 0.99$ ) in the concentration ranges studied. The intra- and inter-day coefficients of variation for the metabolites were tested by measuring standards (25 µM) in blank microme. This test was performed in triplicates and repeated three times on three different days.

### **2.11 *In vivo* Pharmacokinetic Study**

#### **2.11.1 Chemicals**

MDZ free base powder was a generous gift from Hoffman La Roche, NJ, US and internal standard flurazepam (1 mg/ml in methanol) was purchased from Radian International, TX, US. All other chemicals were purchased either from Sigma, MO, US



or Fisher Scientific Incorporate, ON, Canada. All chemicals were analytical reagents and solvents were HPLC grade.

### **2.11.2 Procedure**

Twelve Sprague-Dawley rats were randomly assigned to two groups: the control rats received saline infusion while having free access of rodent chow; and the PN-treated animals received PN infusion only. The treatment lasted for seven days. On the eighth day, all infusions were stopped. The rats were weighed and body weights recorded. MDZ powder was dissolved in a mixture of 20% ethanol, 20% PEG400 and 60% water to give a final concentration of 3 mg/ml. The solution was filtered through a 0.22 µm microfilter. Before giving the drug to the rats, 250 µl of blood was withdrawn from the CA as blank. Then the filtered MDZ solution was given as an *iv* bolus (3 mg/kg) through the JV. A 250 µl volume of blood was taken at 2.5, 5, 7.5, 10, 15, 30, 45, 60 minutes post-dose and a 400 µl volume of blood was withdrawn at 90, 120, and 180 minutes. The blood samples were collected into pre-heparinized Eppendorf tubes (Fisher Scientific Corporation, ON, Canada). The blood samples were centrifuged at 1000 g immediately and plasma was harvested. The plasma samples were stored at – 20° C until analysis.

### **2.11.3 HPLC Assay**

#### **2.11.3.1 Instrumentation and Chromatographic Conditions**

The HPLC system (Waters, MO, US) consisted of a Waters 501 HPLC pump, a Waters 712 WISP automatic injector and a Waters Lambda-Max model 480 LC spectrophotometer detector. The separation of MDZ from other endogenous constituents in plasma samples was achieved using a reverse-phase ZORBAX SB-C18, 4.6 mm ID x



15 cm, 3.5  $\mu$  column (Phenomenex Corporation, CA, US). Mobile phase was a mixture of 60% methanol with 40% double distilled water, and the pH of the mobile phase was adjusted to 3.65 using 1% perchloric acid. The pump flow rate was set at 0.9 ml/min. Signals were detected at 254 nm and integrated using the Baseline 820 software (Dynamic Solutions, Division of Millipore, CA, US). The retention times for internal standard flurazepam and MDZ were 2.3 and 3.6 minute, respectively.

#### **2.11.3.2 Sample Preparation and Extraction**

To quantify the concentration of MDZ in plasma, a 100  $\mu$ l plasma sample was mixed with 100  $\mu$ l of internal standard (flurazepam, 400 ng/ml) solution, and 100  $\mu$ l of 0.1 N sodium hydroxide (NaOH) in a glass tube (16 x 100 mm, Kimax<sup>®</sup>, IL, US). The mixture was vortexed for 30 seconds, followed by the addition of 4ml of diethyl ether. The tube was shaken vigorously for 15 minutes and centrifuged at 1000 g for another 15 minutes. The top organic layer was transferred into a clean test tube and dried under a gentle stream of nitrogen. The samples were finally reconstituted with 100  $\mu$ l of 20% methanol and 70  $\mu$ l of it was injected into the HPLC system.

#### **2.11.4 Data Analysis**

Calibration curves were used to quantify MDZ in plasma samples after an *iv* bolus dose of 3 mg/kg was given to rats. The concentration range of calibration curves was from 31.2 to 2000 ng/ml. The standard solutions were prepared by serially diluting standard MDZ stock solution into five different concentrations. These diluted standards were spiked into blank plasma. The spiked standards were extracted in exactly the same way as those for study samples. The standard curves were constructed by plotting the



peak area ratios of MDZ to the internal standard against standard concentrations. The calibration curves were all linear ( $r^2 > 0.99$ ) in the concentration range studied. The intra- and inter-day coefficients of variation for the procedure were evaluated using spiked plasma MDZ solutions. The samples were prepared in triplicate and the test was repeated three times on three different days. The extraction efficiency was evaluated by comparing the peak area ratio of the same methanolic solution of MDZ with those of spiked plasma.

Concentrations of MDZ in plasma were determined using standard curves. Samples with concentration higher than 2000 ng/ml were diluted with blank plasma. Twice as much sample volume was used to quantify samples with concentrations lower than the lowest limit of the standard curve. The pharmacokinetic parameters were calculated using WinNonlin software (PharSight Corporation, NC, US).

## 2.12 Statistical Analysis

A complete random block design was used in the current project. For *in vivo* study, the pharmacokinetic parameters of MDZ were analyzed using WinNolin software (PharSight Corporation, NC, US) and a Michaelis-Menten equation (WinNonlin, PharSight Corporation, NC, US) was used for estimating enzyme kinetic parameters *in vitro*.

Data were initially examined for normal distribution (Shapiro-Wilk test) and variance homogeneity (Levene test). If the data were judged to be homogeneously distributed, a two-tailed *Student* t-test was performed to evaluate significant differences between the control group and the PN-treated group. When a parameter could not correct its deviation from normality ( $p < 0.05$ ), the parameter was subject to non-parametric tests



(Kruskal-Wallis and Mann-Whitney). The significant level was set at  $p \leq 0.05$ . All results are reported as mean  $\pm$  SD.



## 3 Results

### 3.1 Animal Body and Liver Weight Changes

All animals that participated in this project were healthy and had body weights ranging from 230 to 270 g at the beginning of experiments. Animals in both the chow-fed control group and the PN-treated group gained weight. Although control group rats gained more weight per day compared to that of PN treated animals, there was no statistical significance (Table 3.1). No statistical difference of liver weight or liver weight expressed as percentage of body weight was found between the two groups (Table 3.1).

### 3.2 Endotoxin Level in Rat Serum

Figure 3.1 shows the linearity of a representative standard curve for serum endotoxin with concentrations ranging from 8.3 to 125 pg/ml. Correlation coefficient ( $r^2$ ) values for all standard curves are higher than 0.98. The values for inter- and intra-day coefficient variation (CV) are less than 12%. The detectable limit for endotoxin is less than 1 pg/ml.

After PN treatment, portal serum endotoxin levels were significantly higher compared to those of the controls ( $74 \pm 11$  pg/ml vs  $21 \pm 25$  pg/ml,  $p < 0.05$ ,  $n=6$  for each group). However, the IVC serum endotoxin levels were almost the same between the PN and the control groups ( $35 \pm 34$  pg/ml vs  $38 \pm 36$  pg/ml,  $p > 0.05$ ,  $n=6$  for each group) (Figure 3.2). No endotoxin was detected in any of the liver cytosol samples.



### 3.3 Cytokine Levels in Rat Liver Cytosol

Figures 3.3 to 3.5 are representative standard curves of TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , respectively. Concentrations employed to prepare these standard curves ranged from 15.6 up to 2000 pg/ml. These standard curves are linear ( $r^2 > 0.98$ ). The inter- and intra-day variations are less than 10%. The detectable limits for TNF- $\alpha$ , IL-6 and IL-1 $\beta$  are 4, 8 and 3 pg/ml, respectively.

In liver cytosol, concentrations of TNF- $\alpha$  in PN treated rats were significantly higher than those of controls ( $1.9 \pm 0.84$  ng/ml vs  $0.86 \pm 0.26$  ng/ml,  $p < 0.05$ ,  $n = 6$  for each group). For IL-6, the mean level in PN treated rats was almost three times higher than that of the control ( $3.4 \pm 0.13$  ng/ml vs  $1.2 \pm 0.54$  ng/ml,  $p < 0.05$ ,  $n = 6$  for each group). Levels of IL-1 $\beta$  in liver cytosol, however, were not significantly different between groups,  $0.72 \pm 0.44$  ng/ml for the PN group and  $0.50 \pm 0.20$  ng/ml for the control ( $p > 0.05$ ,  $n = 6$  for each group) (Figure 3.6).

From the plasma data, a PN treatment effect is not obvious. The reason is that 80% of serum cytokine levels were below quantifiable limits (Table 3.2).

### 3.4 Nitrite Levels in Rat Liver Cytosol

The concentrations of nitrite, the end product of NO, in liver cytosol samples were measured using the Griess Reagent method (Arias-Diaz et al 1996). A representative linear standard curve with concentrations ranging from 62.5 to 375 nM is shown in Figure 3.7 ( $r^2 > 0.99$ ).



The nitrite concentrations in liver cytosol samples are shown in Figure 3.8. After a 7-day PN treatment, nitrite levels were almost doubled when compared to those of the controls ( $169 \pm 37$  vs  $268 \pm 37$  nM,  $p < 0.05$ ,  $n = 6$  for each group).

### **3.5 *In Vitro* Microsome Study**

#### **3.5.1 Protein Content in Rat Liver Microsome**

A calibration curve with BSA concentrations ranging from 50 to 500  $\mu$ g/ml was used to estimate protein content in rat liver microsomes. The standard curve is linear ( $r^2 > 0.99$ ) and shown in Figure 3.9. All samples were measured in triplicates.

The mean protein content ( $\pm$  SD) in liver Microsome preparations after PN treatment was  $12.95 \pm 1.68$  mg/g, which was approximately 25% lower than that in the control ( $17.05 \pm 2.46$  mg/g,  $p < 0.05$ ,  $n = 7$  for each group) (Table 3.3).

#### **3.5.2 Total Activities of CYP in Rat Liver Microsomes**

The mean total activity of CYP in livers of rats that received PN treatment was approximately 25% lower when compared to that of the controls ( $0.296 \pm 0.093$  nmol/min/mg vs  $0.396 \pm 0.054$  nmol/min/mg,  $p < 0.05$ ,  $n = 7$  for each group) (Table 3.3).

#### **3.5.3 Formation of Testosterone Metabolites in Rat Liver Microsomes**

The standard curves for the five testosterone metabolites 6 $\beta$ -, 7 $\alpha$ -, 16 $\alpha$ -, 16 $\beta$ - and 2 $\alpha$ -hydroxytestosterone with concentrations ranging from 1  $\mu$ M to 40  $\mu$ M are linear ( $r^2 > 0.98$ ) and are shown through Figures 3.10 to 3.14.

Recovery was evaluated by spiking authentic metabolite standards into the microsomal incubation system without NADPH. These samples were extracted the same



manner as that used for the *in vitro* metabolism samples (Table 3.4). Representative chromatograms of testosterone and its metabolite standards in methanol and that in blank microsomes are shown in Figures 3.15 and 3.16, respectively. Figure 3.17 shows a representative chromatogram of a blank microsome extract containing internal standard, which clearly shows that with the HPLC procedure used, there was no peaks that interfered with those of the metabolites. The detectable limits for 6 $\beta$ -, 7 $\alpha$ -, 16 $\alpha$ -, 16 $\beta$ - and 2 $\alpha$ -hydroxytestosterone were 0.05, 0.05, 0.05, 0.1 and 0.1  $\mu$ M, respectively. Figure 3.18 is a representative chromatogram of an extract of a liver microsomal sample incubated with testosterone. Metabolite formation rates as a function of testosterone concentration were determined using the Michaelis-Menten equation

$$V = \frac{V_m * [S]}{K_m + [S]}$$

Where V is the metabolic turnover rate (nmol/minute/mg);  $V_m$  is the maximum metabolic turnover rate; [S] is the substrate concentration ( $\mu$ M);  $K_m$  is the Michaelis-Menten constant ( $\mu$ M), the substrate concentration required to reach half of the value of  $V_{max}$ .

Seven rats were involved in each study group. It was found there was one potential outlier in each group. In order to decide if these data were outliers, the Q-test as described in the following equation was applied:

$$Q_n = \frac{|X_a - X_b|}{R}$$

Where, R was the range of all data points,  $X_a$  was the suspected outlier,  $X_b$  was the data point closest to  $X_a$  and n was the replication. The 90% confidence interval Q for n = 7



was 0.51. The Q-test proved that Q values for both sets of data were greater than 0.51. This indicates that these two sets of data were outliers and were excluded from analysis.

In general, after PN treatment, the values of  $K_m$  were higher for the formation of all five metabolites (Table 3.5). These values did not achieve statistical difference except the formation of 7 $\alpha$ -hydroxytestosterone. After PN treatment,  $V_{max}$  values were lower for all the metabolites measured.  $V_{max}$  values for 7 $\alpha$ -, 16 $\alpha$ - and 2 $\alpha$ -hydroxytestosterone were 69%, 44% and 37% of the control levels, respectively ( $p < 0.05$   $n=6$  for each group). Although the reductions of  $V_{max}$  for 6 $\beta$ - and 16 $\beta$ -hydroxytestosterone were 23% and 18%, respectively, they didn't reach statistical difference ( $p > 0.05$   $n=6$  for each group) (Table 3.5).

### 3.6 Immunoblotting Results

Western Blot was performed to qualitatively study the impact of PN treatment on individual CYP isozymes. Figure 3.20 shows the expression changes of CYPs 3A2 and 1A1/2 after PN treatment. The bands in the control group for CYP3A2 was slightly thicker and darker than those in the PN group, suggesting that there was a mild reduction of CYP3A2 expression after PN treatment. There was no visible difference observed for the bands of CYP1A1 between the two groups, indicating that there was no significant suppression of CYP1A1 expressions induced by PN treatment.

Figure 3.21 shows the Western Blot results for CYPs 2C11 and 2B1. For CYP2C11, the intensity of bands in the PN treated group was visibly thinner and lighter than those of the controls, indicating that the expression of CYP2C11, one of the major CYP isoforms in rat liver, has been reduced as a result of PN treatment. However, the change observed in CYP2B1 was not consistent among animals. The band from one rat in



the PN group (Lane 1) was slightly darker compared to those in the control group (Lanes 6,7,8), whereas the other two (Lanes 2,3) showed similar intensity. This could be due to variable response to the PN treatment.

Overall, it was concluded that protein expression of several major CYP isoforms in rat liver had been compromised after PN treatment.

### **3.7 Rat Live Histology Examination**

Representative diagrams of liver histology from a control rat and a PN-treated rat are shown in Figures 3.21 and 3.22, respectively. The number of activated Kupffer cells is similar in the PN-treated and control rats (Table 3.6). However, the number of activated endothelial cells and circulating neutrophils in the PN-treated rats are visibly higher (Figure 3.23) when compared to those of the controls (Figure 3.22). These results suggested that the use of Kupffer cell in the liver as an indicator resulting from portal endotoxin stimulation in the current animal model is not appropriate.

### **3.8 Pharmacokinetic Study of Midazolam**

A linear standard curve of MDZ in plasma with concentrations ranging from 31.2 pg/ml to 2000 pg/ml is shown in Figure 3.24 ( $r^2 > 0.99$ ).

Recovery of MDZ from plasma was evaluated by comparing extracts of MDZ spiked in blank plasma to those of MDZ dissolved in methanol (Table 3.7). Representative chromatograms of blank plasma and standard MDZ spiked in plasma are shown in Figures 3.25. No interfering peaks were detected under the employed HPLC conditions. A representative chromatogram of MDZ in rat plasma after the animal received a 3mg/kg *iv* bolus dosage is shown in Figure 3.25.



Reproducibility of the assay was validated using quality control samples. The inter- and intra- day coefficients of variation are less than 10% (Table 3.8). The detectable limit for MDZ is 20 pg/ml.

The pharmacokinetic parameters of MDZ were calculated using WinNonlin (PharSight, CA, US) . The plasma concentration-time profile of MDZ is best described by a bi-exponential equation following an *iv* bolus dose

$$C(t) = A * e^{-\alpha t} + B * e^{-\beta t}$$

Where C (t) is the concentration at time t, the constants,  $\alpha$  and  $\beta$ , are rate constants for the distribution phase and elimination phase, respectively. The constants A and B are the y intercepts for each exponent. Total body clearance ( $CL_{total}$ ), elimination half-life ( $T_{1/2\beta}$ ) and volume of distribution at steady-state ( $V_{ss}$ ) were calculated using standard methods (Gibaldi & Perrier 1982).

MDZ plasma concentration vs time profiles are depicted in Figure 3.26 after a 3mg/kg *iv* bolus dose. In all rats, the concentration-time profiles are best described by a bi-exponential function. A summary of major pharmacokinetic parameters is listed in Table 3.9. PN treatment prolongs the elimination half-life of MDZ from 35 to 49 minutes ( $p > 0.05$ ) and significantly decreases the clearance from 117 to 67.2 ml/min/kg ( $p < 0.05$ ). The mean AUC value of PN treated rats is approximately two times higher than that of the control ( $p < 0.05$ ). The mean values of volume distribution (4.2 ml/kg for the control and 3.8 ml/kg for PN treated rats) are almost identical.



**Table 3.1 Mean ( $\pm$  SD) body weight and liver weight changes after rats received a 7-day PN treatment**

	Liver wight <sup>1</sup> (g)	Percentage <sup>2</sup> (%)	Body weight change (g/day)
Control <sup>3</sup>	10.98 (0.81) <sup>5</sup>	3.79 (0.44)	6.88 (2.70)
PN <sup>4</sup>	10.39 (0.99)	4.25 (0.39)	4.04 (1.08)

1. Liver weight at the end of treatment
  2. Liver weight presented as percentage of body weight at the end of treatment
  3. Control group rats were fed with normal rodent chow and received infusion of saline
  4. Parenteral nutrition treatment group rats received infusion of parenteral nutrition solution only
  5. Data presented as “average (SD)”
- N = 8 for each group  
Significant level at  $p < 0.05$



Table 3.2 Serum cytokine levels from rats that received a 7-day PN treatment

Sample	Portal Vein (pg/ml)			Inferior vena cava (pg/ml)		
	TNF- $\alpha$	IL-6	IL-1 $\beta$	TNF- $\alpha$	IL-6	IL-1 $\beta$
Control						
1	5	5	ND	5	ND	ND
2	22	ND	ND	24	ND	ND
3	ND	ND	ND	ND	ND	ND
4	ND	47	ND	ND	13	ND
5	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND
PN						
1	ND	ND	ND	ND	ND	ND
2	31	ND	ND	36	ND	ND
3	ND	ND	ND	6	ND	ND
4	6	10	ND	24	12	ND
5	ND	ND	ND	ND	20	ND
6	ND	12	ND	ND	ND	ND

ND = not detectable



**Table 3.3 Protein contents and total cytochrome P450 (CYP) activities in livers from rats that received a 7-day PN treatment (\*  $p < 0.05$ ,  $n = 7$  for each group). Protein contents were measured using Bradford's method and BSA was used as the standard. The total activities of CYP were measured using CO binding method.**

	Protein Content		Total activity of CYP	
	mg/g	SD	nmol/min/mg	SD
PN-treated	12.95*	1.68	0.296*	0.093
control	17.05	2.46	0.396	0.054



Table 3.4 Percent recovery of testosterone and its 6β-, 7α-, 16α-, 16β-, and 2α-hydroxylated metabolites in rat liver microsomes

Spiked concentration 0.5 μM						
Sample	6β	7α	16α	16β	2α	testosterone
1	94.2	105.9	101.2	103.8	98.2	109
2	92.8	106.7	107.3	103.6	100.1	109.5
3	102.8	106.9	105.3	108.5	105.2	108.3
4	101.9	108.2	106.1	107.6	103.8	110.6
Average	97.9	106.9	105	105.9	101.9	109.4
SD	5.1	1	2.5	2.5	3.2	1
CV (%)	5.3	0.9	2.4	2.4	3.1	0.9

Spiked concentration 1.0 μM						
Sample	6β	7α	16α	16β	2α	testosterone
1	95.6	102.8	100.4	105.7	97.4	112.3
2	99.7	104	99.5	105	99.4	114.5
3	100.2	103.3	101.7	105.2	100.9	103.8
4	96.5	101.9	101.4	102.7	96.2	106.3
Average	98	103	100.7	104.6	98.5	109.2
SD	2.3	0.9	1	1.3	2.1	5
CV (%)	2.3	0.9	1	1.3	2.1	4.6



**Table 3.5 Effects of a 7-day PN treatment on rat liver microsomal metabolism of testosterone. Michaelis-Menten parameters ( $\pm$  SD) were measured for comparison**

$V_{\max}$ (nmol/mg/min)		
Hydroxylated Metabolite	PN-treated	control
6 $\beta$ -	1.95 (0.99)	2.55 (1.12)
7 $\alpha$ -	0.37 (0.09)*	0.54 (0.12)
16 $\alpha$ -	1.09 (0.63)*	2.47 (0.94)
16 $\beta$ -	0.11 (0.02)	0.13 (0.04)
2 $\alpha$ -	1.03 (0.63)*	2.81 (1.51)

$K_m$ ( $\mu$ M)		
Hydroxylated Metabolite	PN-treated	control
6 $\beta$ -	194 (95)	153 (42)
7 $\alpha$ -	138 (28)*	94 (27)
16 $\alpha$ -	304 (176)	201 (112)
16 $\beta$ -	165 (86)	133 (85)
2 $\alpha$ -	157 (52)	129 (51)

N = 6 for each group  
Substrate concentrations ranged from 10 ~ 750  $\mu$ M  
Incubation condition were at 37<sup>o</sup> C for 15 minutes  
Significant level set at  $p < 0.05$



Table 3.6 Activated Kupffer cells in rat livers after receiving a 7-day PN treatment

Animal	Treatment	Activated Kupffer cell*
1	PN	8
2	PN	5
3	PN	7
4	Control	2
5	Control	6
6	Control	2

\* Total numbers of activated Kupffer cells counted in three separate linear area of 0.475 mm.



**Table 3.7 Recovery of midazolam in rat plasma sample (%)**

Sample	Spike concentration 100pg/ml	Spike concentration 1000pg/ml
1	72	86
2	84	87
3	81	90
4	87	88
Average	81	88
SD	6.5	1.7
CV (%)	8.0	2.0



**Table 3.8 Inter- and intra-day precision of midazolam extraction procedure**

**Intra-day precision**

	Sample 1 (1500 ng/ml)	Sample 2 (750 ng/ml)	Sample 3 (100 ng/ml)
1	1403	689	91
2	1521	832	109
3	1634	772	96
Average	1519	764	98
SD	116	72	9.3
CV (%)	7.6	9.4	9.4

**Inter-day precision**

	Sample 1 (1500 ng/ml)	Sample 2 (750 ng/ml)	Sample 3 (100 ng/ml)
Day 1	1403	689	91
Day2	1438	713	102
Day3	1567	763	110
Average	1469	722	101
SD	86	38	9.5
CV (%)	5.9	5.2	9.4



**Table 3.9 Mean ( $\pm$ SD) pharmacokinetic parameters of midazolam in rats, which received a 7-day PN treatment, after a 3 mg/kg *iv* bolus dose**

	Control	PN
$T_{1/2\alpha}$ (minute)	4.06 (0.89)	5.02 (3.24)
$T_{1/2\beta}$ (minute)	34.8 (12.2)	48.9 (11.2)
$AUC_{0 \rightarrow \infty}$ (minute.mg/ml/kg)	110 (45)	202 (34)*
$V_{ss}$ (l/kg)	4.20 (0.66)	3.76 (0.44)
$CL_{total}$ (ml/minute/kg)	117 (30)	67.2 (17.1)*

N = 6 for each group

\*  $p < 0.05$



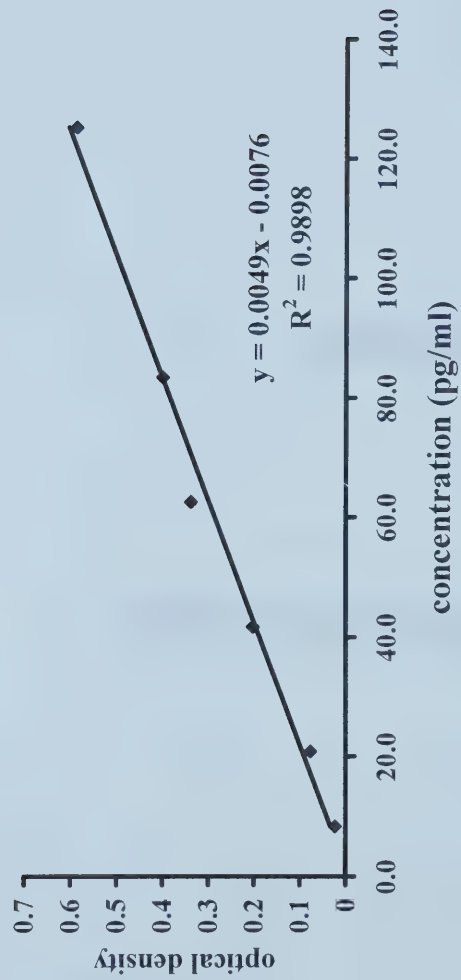
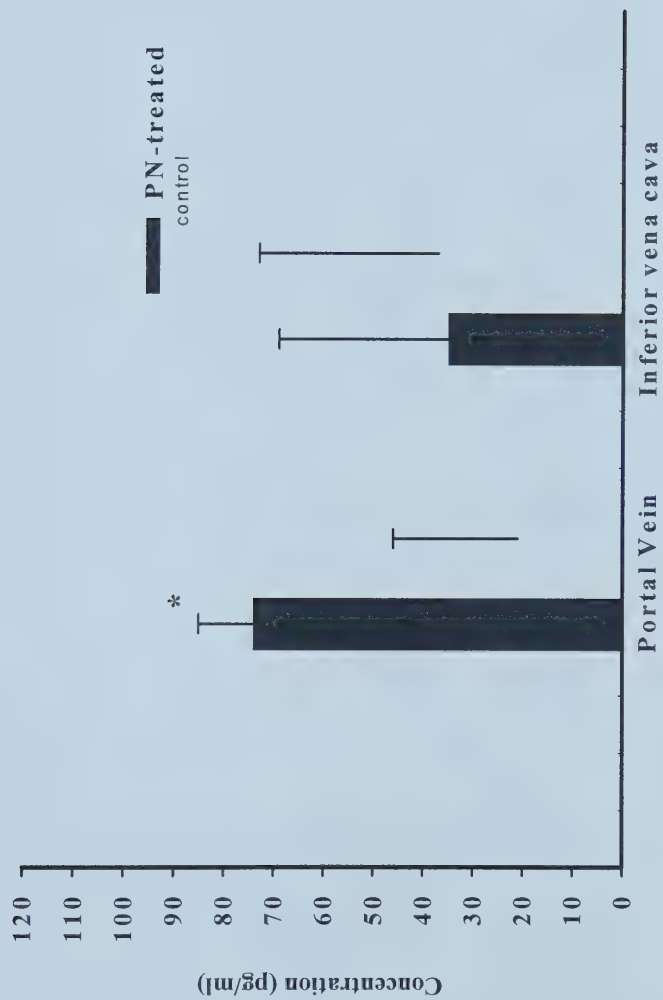


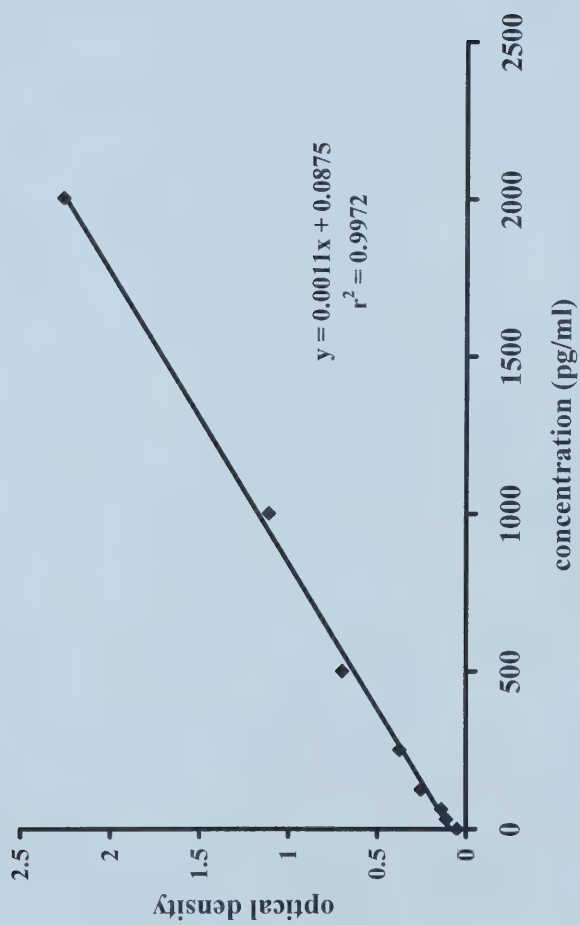
Figure 3.1 A representative standard curve of endotoxin in rat plasma (n=3)





**Figure 3.2** Serum concentrations of endotoxin from rats that received a 7-day PN treatment (\* $p < 0.05$ ,  $n = 6$  in each group)





**Figure 3.3** A representative standard curve of TNF- $\alpha$  in rat plasma (n=3)



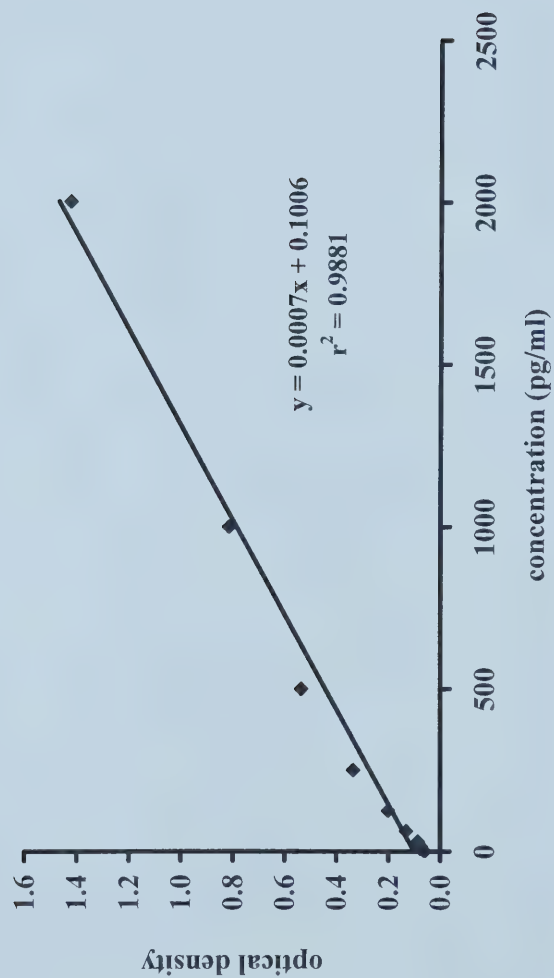


Figure 3.4 A representative standard curve of IL-6 in rat plasma (n=3)



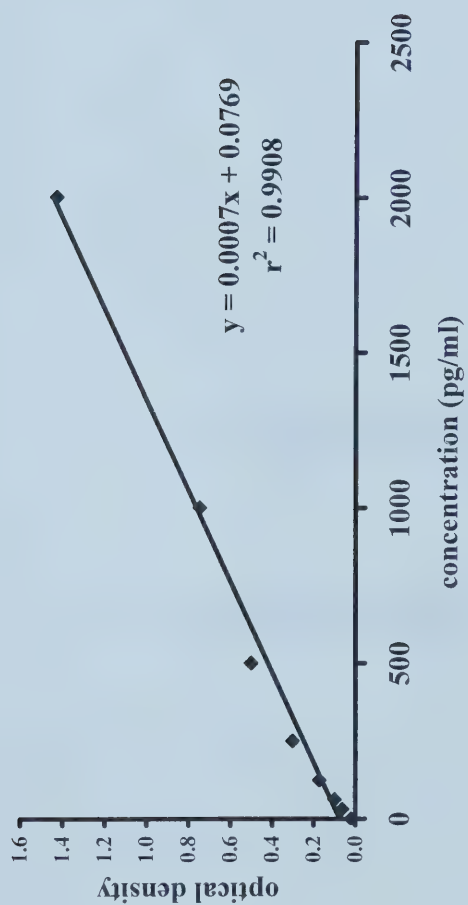


Figure 3.5 A representative standard curve of IL-1 $\beta$  in rat plasma (n=3)



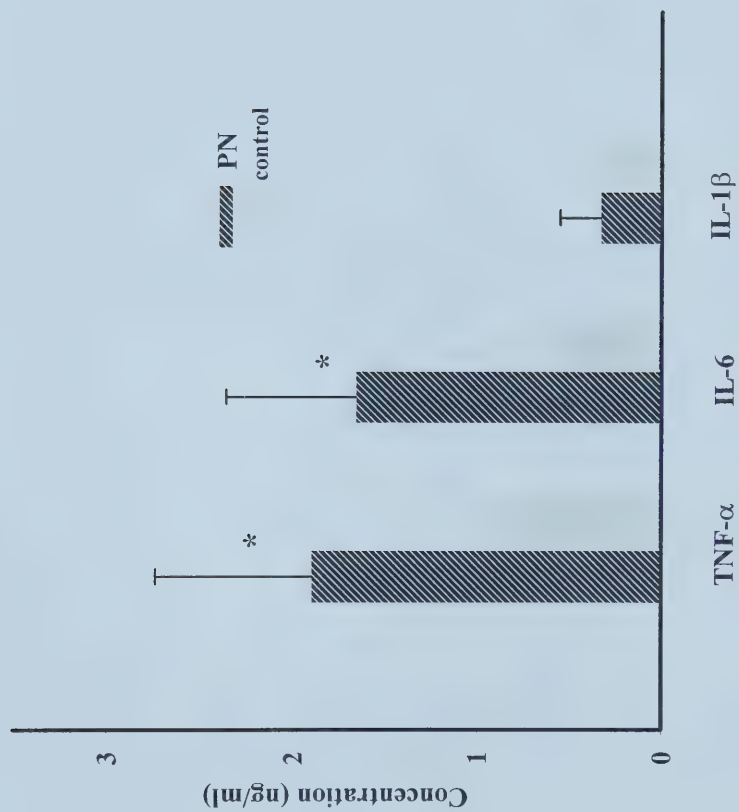


Figure 3.6 Cytokine concentrations in liver cytosol collected from rats after a 7-day PN treatment (\* $p < 0.05$ ,  $n = 6$  in each group)



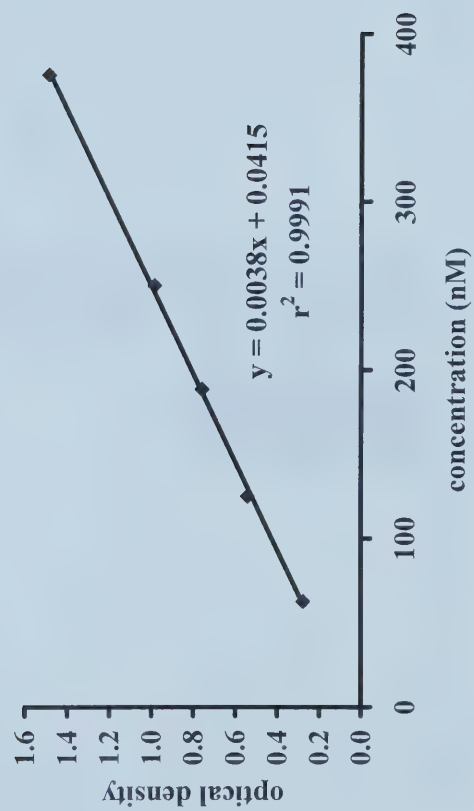


Figure 3.7 A representative standard curve of nitrite in rat liver cytosol (n=3)



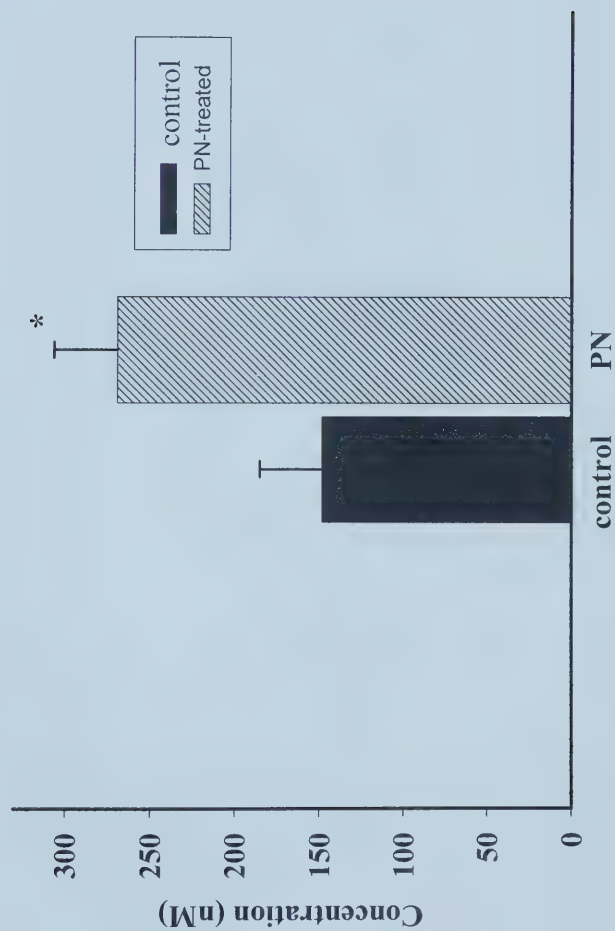
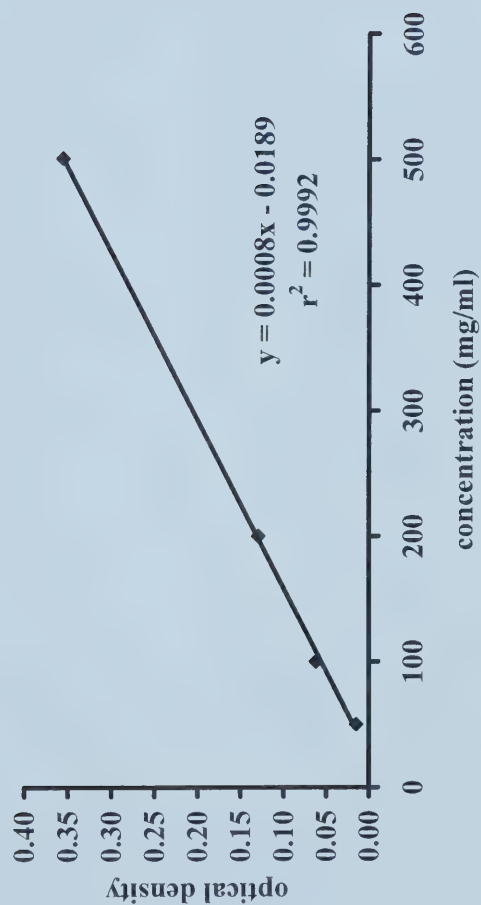


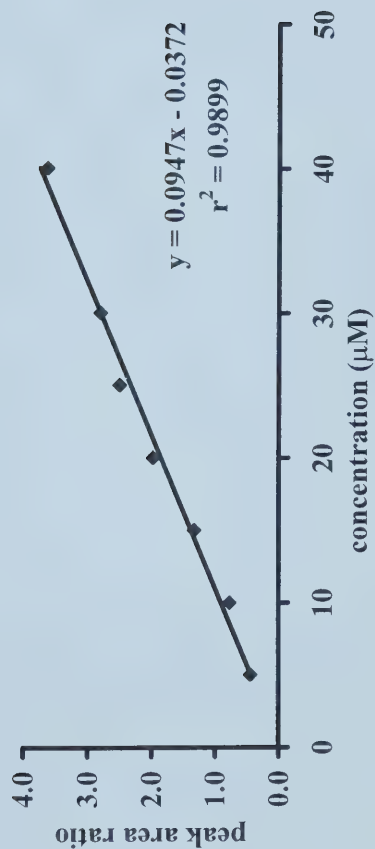
Figure 3.8 Nitrite concentrations in liver cytosol from rats that received a 7-day PN treatment (\*  
 $p < 0.05$ ;  $n = 7$  in each group)





**Figure 3.9** A representative standard curve of protein content measured using the Bradford method. (n = 3, BSA was used as a standard)





**Figure 3.10** A representative standard curve of 6 $\beta$ -hydroxytestosterone in a rat liver microsome preparation, plotted as the peak area ratios of the 6 $\beta$ -hydroxytestosterone to the internal standard against its concentrations (n=3)



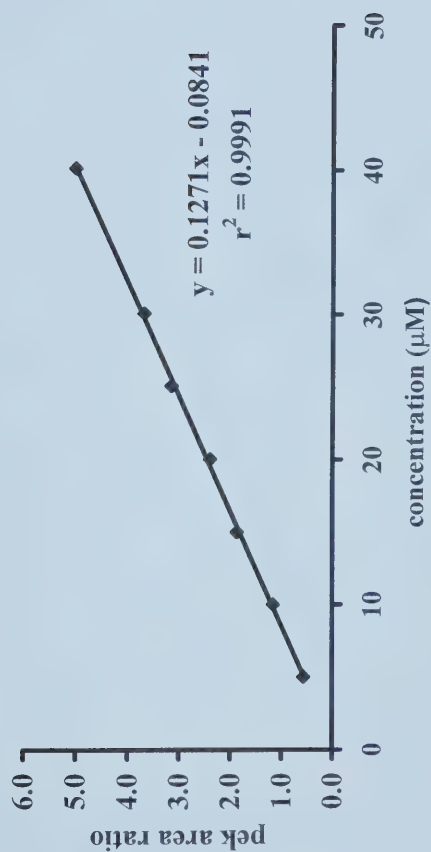


Figure 3.11 A representative standard curve of 7 $\alpha$ -hydroxytestosterone in a rat liver microsomal preparation, plotted as the peak area ratios of the 7 $\alpha$ -hydroxytestosterone to the internal standard against its concentrations (n=3)



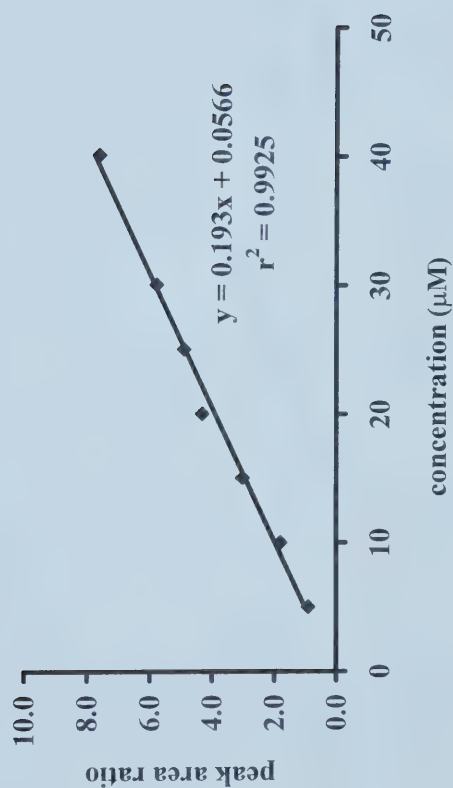
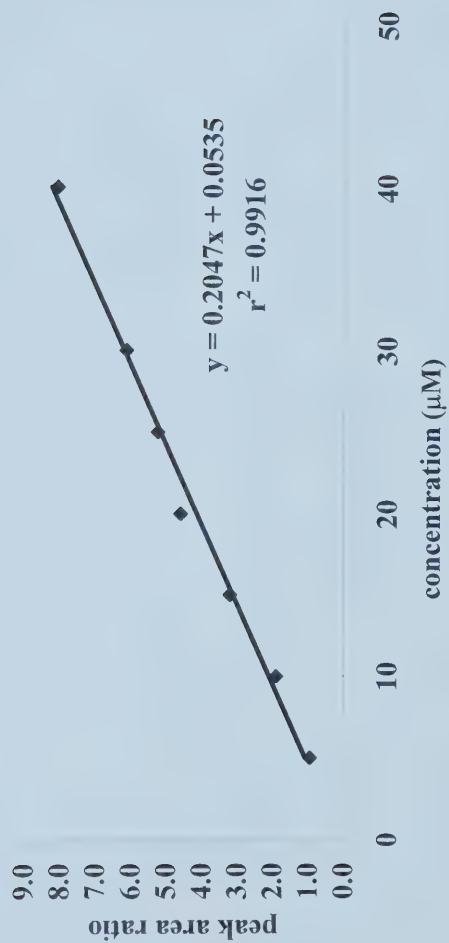


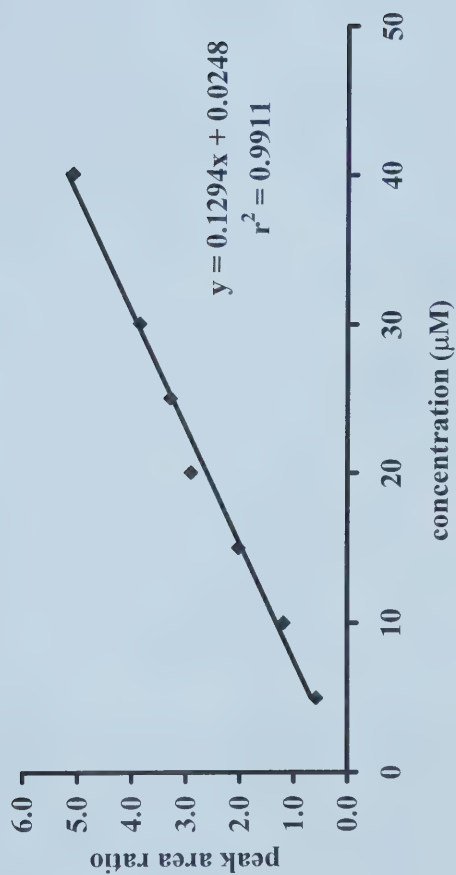
Figure 3.12 A representative standard curve of 16 $\alpha$ -hydroxytestosterone in a rat liver microsome preparation, plotted as the peak area ratios of the 16 $\alpha$ -hydroxytestosterone to the internal standard against its concentrations (n=3)





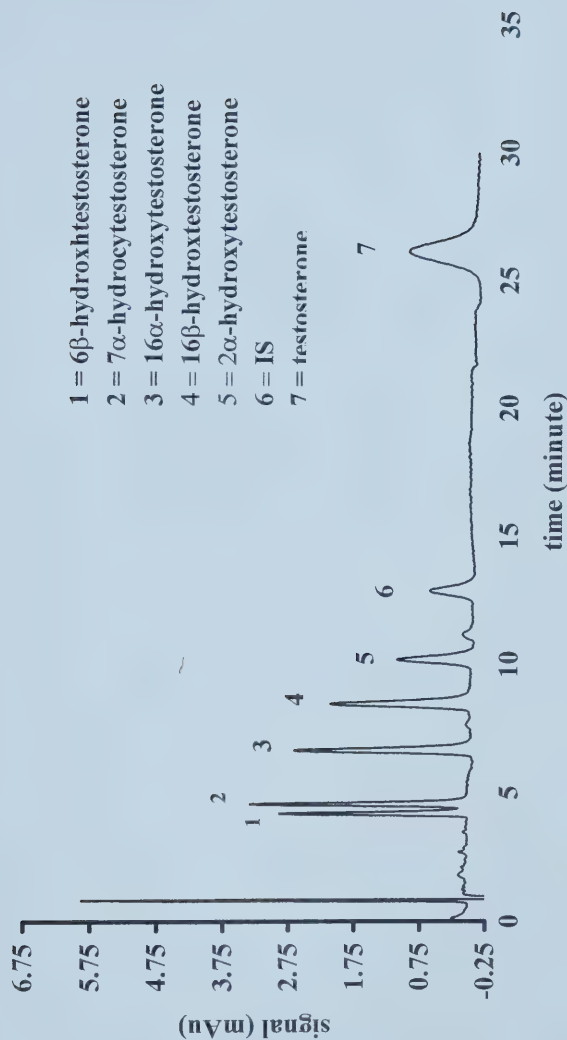
**Figure 3.13** A representative standard curve of 16 $\beta$ -hydroxytestosterone in a rat liver Microsome preparation, plotted as the peak area ratios of the of 16 $\beta$ -hydroxytestosterone to the internal standard against its concentrations (n=3)





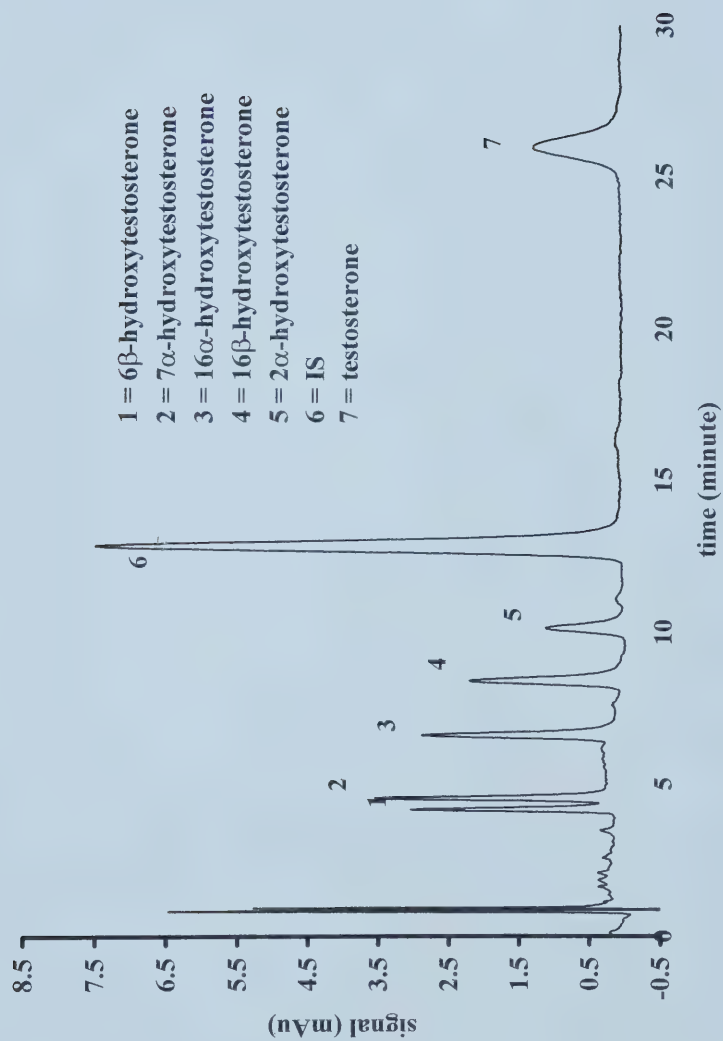
**Figure 3.14** A representative standard curve of 2 $\alpha$ -hydroxytestosterone in a rat liver microsome preparation, plotted as the peak area ratios of the 2 $\alpha$ -hydroxytestosterone to the internal standard against its concentrations (n=3)





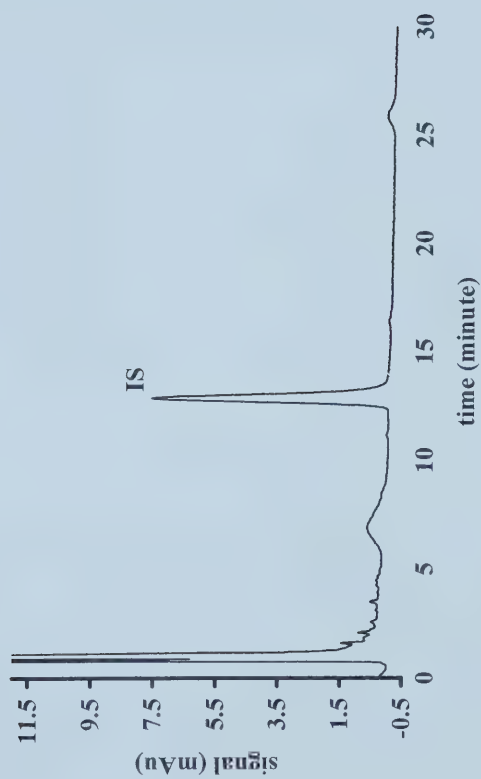
**Figure 3.15** A representative HPLC chromatogram of a standard methanolic solution of testosterone and its metabolites samples (the peaks were separated by using a reverse-phase Eclipse XDB-C8 4.6mm ID X 7.5 cm, 3.5 micron column; the mobile phase was consisted of 43% methanol : 57% water; the flow rate was set at 1.0 ml/min at 40 $^{\circ}$ C; UV signal was monitored at 247nm; the retention times for testosterone, IS, 6 $\beta$ -, 7 $\alpha$ -, 16 $\alpha$ -, 16 $\beta$ - and 2 $\alpha$ -hydroxytestosterone were 26.3, 12.9, 4.1, 4.5, 6.5, 8.3 and 10.3 minutes, respectively).





**Figure 3.16** A representative HPLC chromatogram of standards (testosterone and its metabolites, 25  $\mu$ M) spiked in blank rat liver microsomes (see Figure 3.15 for HPLC conditions)





**Figure 3.17** A representative HPLC chromatogram of blank rat liver microsomes (see Figure 3.15 for HPLC conditions).



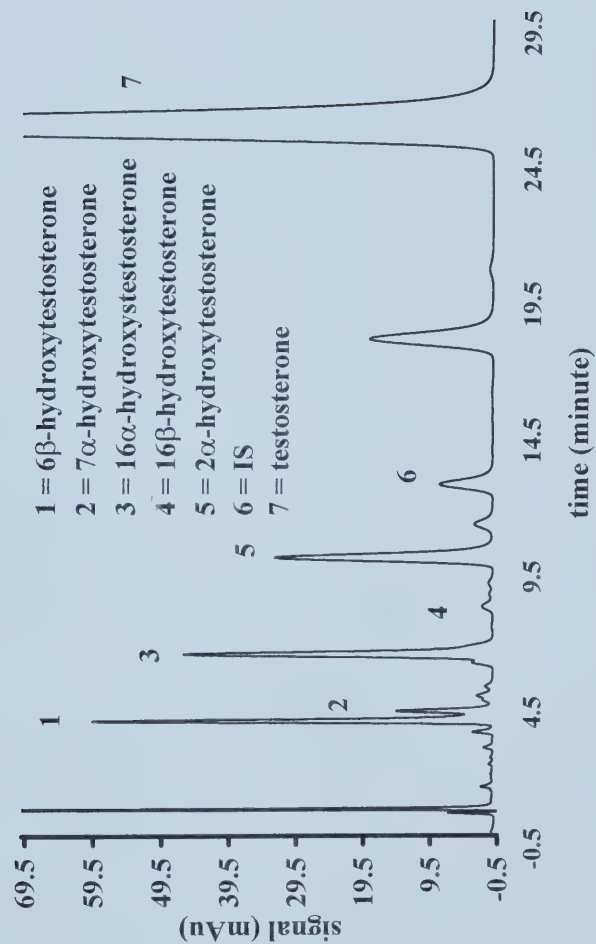


Figure 3.18 A representative HPLC chromatogram of a rat liver microsomal sample incubated with testosterone (250  $\mu$ M) (see Figure 3.15 for HPLC conditions)



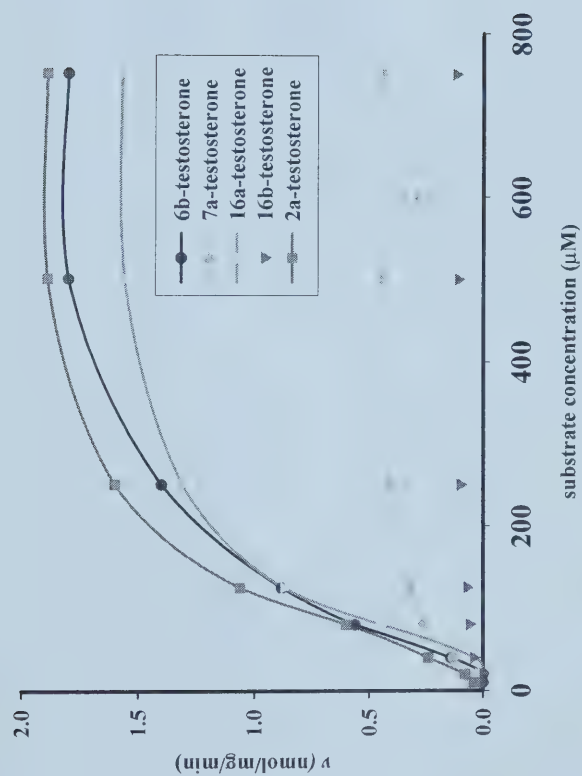
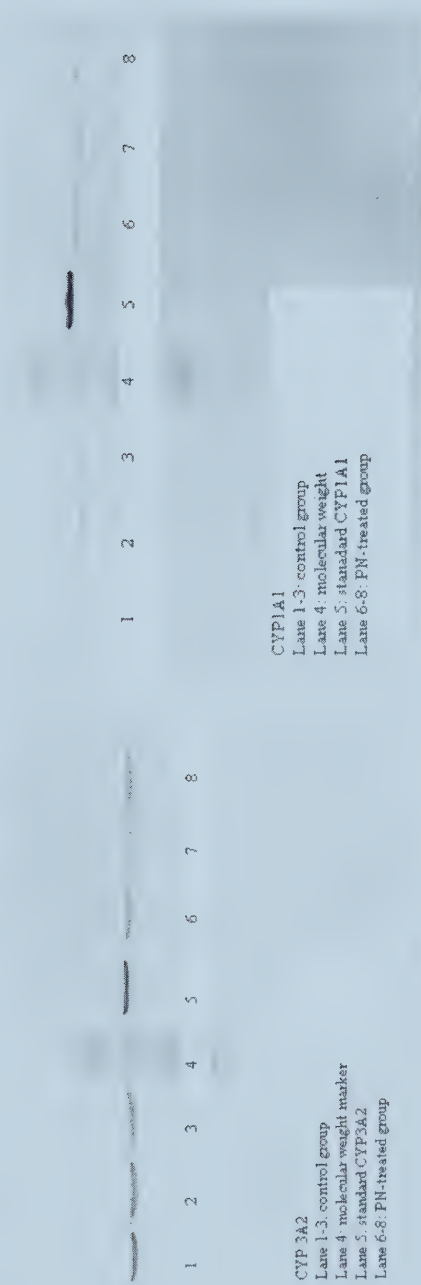


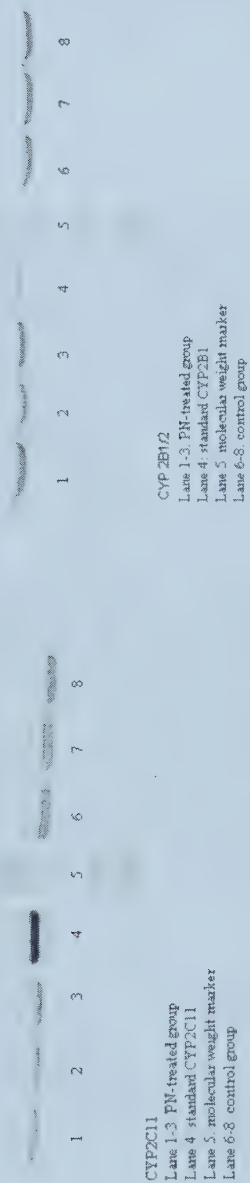
Figure 3.19 A representative diagram of testosterone metabolite formation (substrate concentration ranged from 10 to 750  $\mu\text{M}$ , incubated at 37°C for 15 minutes, n=8 for each group)





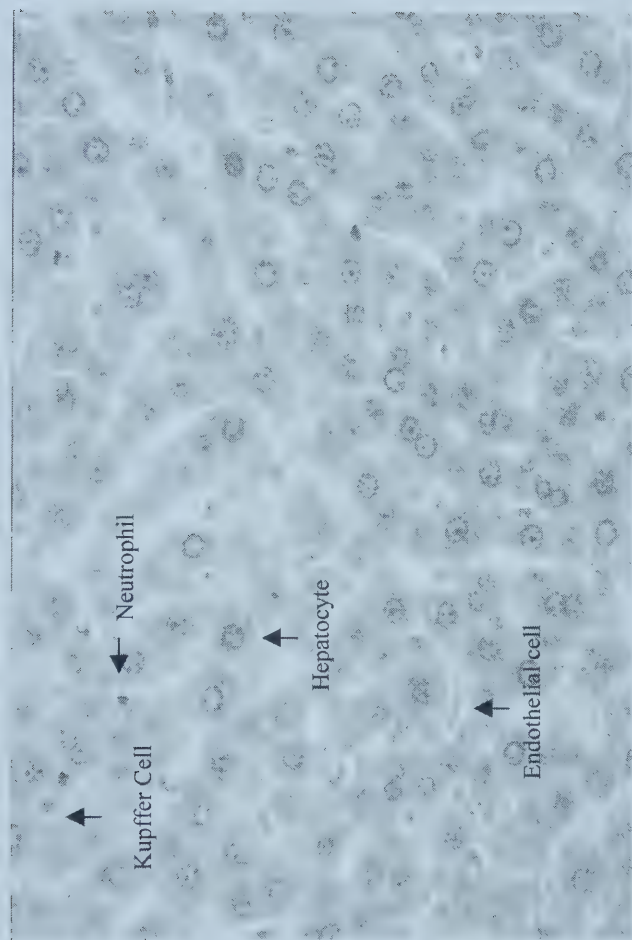
**Figure 3.20** Examination of changes of CYP 3A2 and CYP 1A1/2 content after a 7-day PN treatment using Western blot (microsomal protein was separated by 7.5% SDS polyacrylamide gel and electrophoretically blotted onto nitrocellulose membranes. Levels of CYP proteins in microsomal samples were qualitatively measured using a polyclonal antibody to each individual CYP isoform).





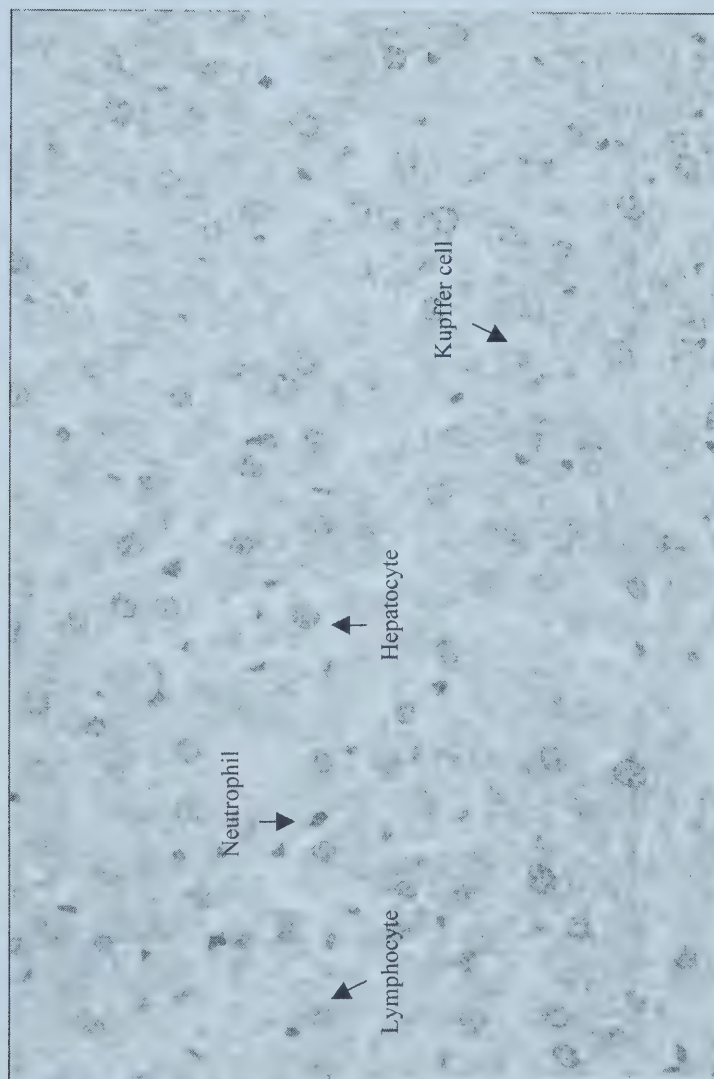
**Figure 3.21 Examination of changes of CYP2C11 and CYP2B1/2 content after a 7-day PN treatment using Western blot (see Figure 3.20 for Western blot conditions)**





**Figure 3.22** A representative picture of liver histology in control rat after a seven-day treatment with saline





**Figure 3.23** A representative picture of liver histology in rat that received a seven-day PN treatment



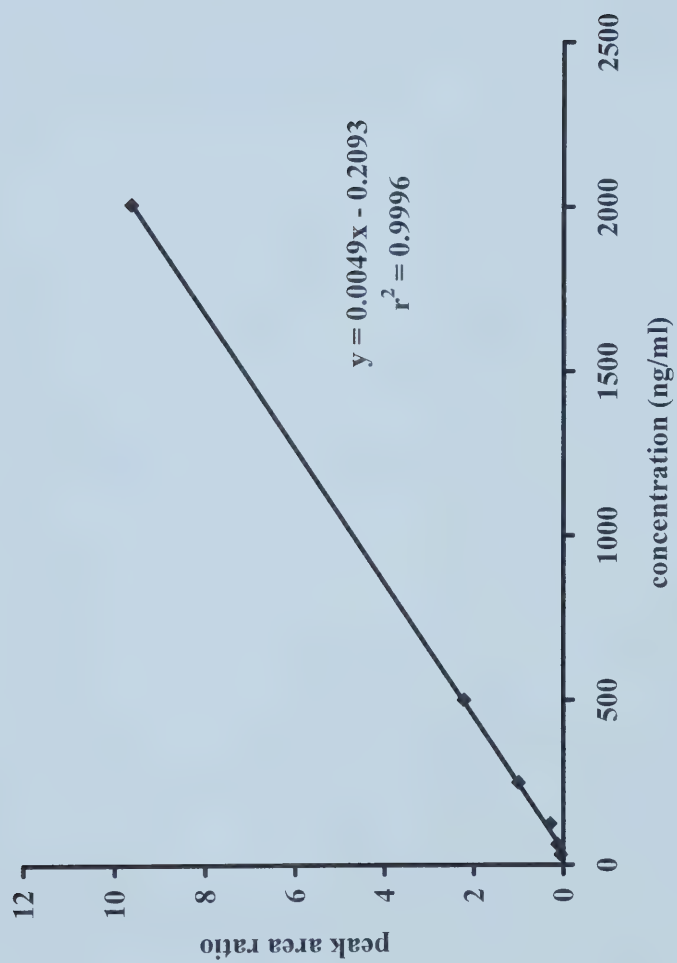
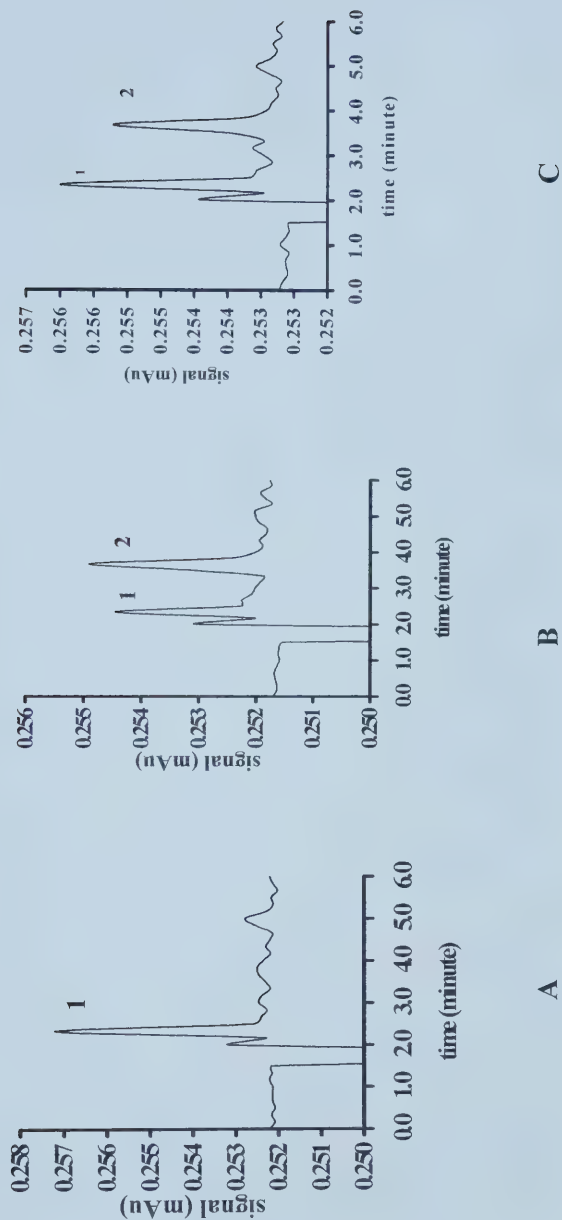


Figure 3.24 A representative standard curve of midazolam in rat plasma, plotted as the peak area ratios of midazolam to internal standard against its concentrations (n=3)





**Figure 3.25** A representative HPLC chromatogram of blank rat plasma (A), standard blank rat plasma (500 ng/ml) spiked with midazolam (B) and midazolam in rat plasma sample after a 3 mg/kg *iv* bolus dose (C). (1 = internal standard, retention time 2.3 minute; 2 = midazolam, retention time 3.6 minute)



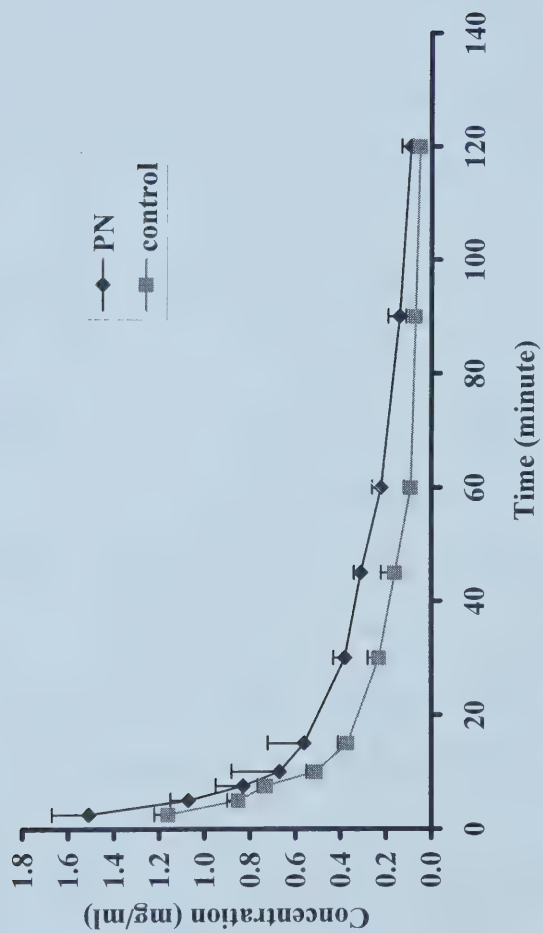


Figure 3.26 Midazolam plasma concentration vs time curves in rat after a 3 mg/kg *iv* dose (n = 6 in each group)



## 4 Discussion

### 4.1 Overview

Since its introduction to clinical practice, PN has become an important nutritional support for patients who are either very ill or require extensive surgery (Cano 2000). The use of PN; however, have been associated with a number of clinical manifestations, primarily gut and liver damage (Matilla et al 2000). There are isolated reports in the literature suggesting that a dormant gut is favorable for the migration of bacteria up to the gut (Deitch et al 1990). This movement, called bacterial translocation, increases the transport of bacteria or dead bacterial cell wall, endotoxin, into the blood circulation. The presence of endotoxin in the general circulation can cause liver injury (Shedlofsky et al 1997). Results from our laboratory and others have shown that CYP isozymes and drug metabolic ability are impaired in animals receiving PN (Ke et al 1990; Earl-Salotti & Charland 1994; Zaman et al 1997). In general, it is well documented that high levels of nitric oxide can cause a reduction in the rate of drug metabolism (Wink et al 1993; Muller et al 1994). The release of certain cytokines is also responsible for the depression of CYP expression and activity (Morgan et al 1994; Chen et al 1995). It is also well known that endotoxin triggers a release of cytokines (Schreiber et al 1982; Baumann et al 1984) and nitric oxide (Nathan 1992; Stuehr & Griffith 1992). Based on this set of information, it is hypothesized that liver injuries induced by PN is caused by bacterial translocation. The sequence of events that happens involves bacterial translocation, which is indicated by an increased level of endotoxin in circulation. The increase in endotoxin level is associated with the release of cytokines and nitric oxide. This sequence of events will lead to an



alteration of drug elimination when the affected CYP isozymes are involved in the disposition of these drugs.

## **4.2 Levels of Endotoxin in Rat Plasma After a 7-day PN Treatment**

Endotoxin, a gram-negative bacteria cell wall, is a stimulus of many proinflammatory cells that release cytokines, such as TNF- $\alpha$ , IL-6 and IL-1 $\beta$ . Endotoxin could activate hepatic Kupffer cell, resulting in liver hypersensitivity to minimal exposures to endotoxin (Nolan 1989). Pappo and his coworkers found that endotoxin acting as a hepatotoxin may induce hepatic steatosis in rats receiving PN (Pappo et al 1991). Intravenous nutrition has been found to impair gut barrier function and enhance endotoxin transport across the gut compared with control animals given pellets enterally (Gonnella et al 1992). Therefore, we chose endotoxin as an index of bacterial translocation after a 7-day PN treatment in a rodent model.

In the endotoxin study, it was found that endotoxin concentration in the portal vein of a PN treated rat was more than three times higher than that in a control rat ( $74 \pm 11$  pg/ml vs  $21 \pm 25$  pg/ml) (Figure 3.2). The jugular level of endotoxin on the other hand did not differ significantly from the control level ( $35 \pm 34$  pg/ml vs  $38 \pm 36$  pg/ml) (Figure 3.2). These data strongly suggest that endotoxin was derived from bacterial translocation.

Other researchers have reported similar findings. Jiang and coworkers reported levels of  $92 \pm 31$  and  $67 \pm 40$  pg/ml of endotoxin in the portal vein and peripheral circulation, respectively in a rat hemorrhagic shock model (Jiang et al 1995). Five of six postoperative dogs showed a measurable, but lower level of endotoxin in peripheral



venous blood when compared to that of the portal system, although the difference was not statistically significant ( $P = 0.06$ ) (Peterson et al 1991). In 56 patients with cirrhosis, huge variabilities in hepatic venous endotoxin concentration ( $73 \pm 110$  pg/ml) and peripheral venous endotoxin concentration ( $31 \pm 58$  pg/ml) were found. All these data, along with ours, support the idea that endotoxin is derived from the gut in the presence of liver disease and a higher level of endotoxin has been found in the portal circulation (Lumsden et al 1988).

No statistical difference was observed in the peripheral circulating system between two groups ( $35 \pm 34$  pg/ml vs  $38 \pm 36$  pg/ml) (Figure 3.2). This could be related to the detoxification function of the liver. Endotoxin is cleared from the circulation mainly by the liver and the spleen (Kleine et al 1985; Freudenberg et al 1992). Uptake by Kupffer cells in the liver represents the first step the body takes to clear gut-derived endotoxin (Fox et al 1987). Once cleared from the portal system, endotoxin is modified and detoxified. This could probably explain why no measurable endotoxin was found in the liver cytosol of PN treated animals.

In the literature, the approach to evaluate the effect of endotoxin on hepatic function was to give endotoxin either by intravenous bolus or infusion. The endotoxin concentration observed in the circulation could reach a level which is as high as the level seen in patients with septic shock ( $110\text{-}726$  pg/ml) (Opal et al 1999). In order to mimic mild liver dysfunction observed in patients receiving PN, we measure endotoxin level in the portal and peripheral circulation after PN treatment without giving any exogenous endotoxin. It's also very important to point out that when a dose of endotoxin is given exogenously, it will trigger bursts of cytokines production. The profile of cytokines



produced is quite different from that observed under a slow and continuous endogenous production of endotoxin, as observed in experimental models of infection (Cross et al 1993).

In the current study, the composition of PN solution was identical to those previously used in our laboratory (Zaman et al 1997). The preparation contains 4.25% amino acid and 25% dextrose plus calcium and multivitamins. Rats receiving such PN solution for seven continuous days showed no significant difference in body weight gains when compared to those in the control animals (Table 3.1). In a previous study, Zaman et al (1997) showed that infusion of this PN solution to rats for seven continuous days was associated with mild hepatic dysfunction, particularly hepatosteatosis. However, the condition was found to have no correlation with the serum liver function test (LFT) markers, indicating liver function test was not sensitive to PN-related hepatic dysfunction under the current nutritional regiment. Therefore, we did not measure the LFT values in the current study.

### **4.3 Level of Cytokines in Rat Liver Cytosol After a 7-day PN**

#### **Treatment**

PN is associated with atrophy of the gut and can cause bacterial translocation (Alverdy et al 1988). Bacterial translocatin could promote endotoxin absorption from the gut into circulation and activate Kupffer cells in the liver. This activation could trigger the release of cytokines.

In the current study, we examined PN-related liver histologic changes, primarily focusing on inflammatory changes, and changes in the number of Kupffer cells (Figures 3.21 and 3.22). The numbers of Kupffer cell counts in each linear area tends to be slightly



higher in PN treated rats. But the difference was not statistically significant (Table 3.6). This is not surprising, because the level of endotoxin measured in the portal vein in PN animals is relatively low when compared with that observed in more severe condition such as sepsis. The inflammation related changes were, however, more dramatic in that the numbers of activated endothelial cells and circulating neutrophils is increased (Figure 3.23).

Although the Kupffer cell is an important cellular mediator of endotoxin-induced liver injury, the leukocyte population, including lymphocytes or monocytes, may also mediate liver injury during endotoxin exposure (Hewett et al 1993). Previous investigations have proven that the presence of an increased numbers of neutrophils in the liver could mediate liver injury by producing a variety of proteases and oxygen radical species that directly or indirectly injure cellular constituents of the hepatocytes. (Heflin & Brigham 1981; Tate & Repine 1983; Brigham & Meyrick 1986; Chang et al 1987).

We also measured various cytokines levels in liver cytosol and found significantly higher level of TNF- $\alpha$  and IL-6 in liver cytosol in PN rats compared with those in the control rats (Figure 3.6). Data in the literatures have shown that endotoxin exerted multiple effects on the liver including induction of acute phase protein and release of various proinflammatory cytokines (Schreiber et al 1982; Baumann et al 1984). This higher level of cytokines in liver cytosol in PN treated rats could be triggered by elevated level of endotoxin measured in the portal vein after a 7-day PN treatment.

Extremely low level of cytokines (5 to 24 pg/ml) could be detected in the serum samples of PN and control rats and the concentration of cytokines in most of the samples



was under detection limits (5 pg/ml) (Table 3.2). The reason for that is not clear. The possible reasons are: 1) cytokines are produced locally, 2) cytokines have extremely short half-lives). It may be that blood is not a good sampling site for studying the production of cytokines in this rodent PN model.

#### **4.4 Levels of Nitrite in Rat Liver Cytosol After a 7-day PN Treatment**

In the present study, the NO concentration, measured as nitrite, was found to be two times higher in the liver cytosol of PN rats than in control animals (Figure 3.8). Higher levels of NO have also been observed in neonates receiving PN (Pitkanen et al 1991).

Although NO is normally produced in relatively small quantities, NO synthesis increases substantially after exposure to immunological stimuli such as bacteria and/or endotoxin (Hibbs et al 1987; Curran et al 1990; Moncada et al 1991; Nathan 1992; Pittner & Spitzer 1992; Stuehr & Griffith 1992). TNF- $\alpha$  and IL-6 are two major proinflammatory cytokines that are released during endotoxin stimulation and have been shown to enhance superoxide production (Jersmann et al 1998). TNF-mediated cytotoxicity is associated with free radical generation (Larrick & Wright 1990). Therefore, the higher level of NO in PN observed in this study may be directly related to increased levels of TNF- $\alpha$  and IL-6.

Previous PN studies have not definitively shown that free radical generation was due to PN administration alone. The presence of other diseases in patients made the distinction impossible (Wispe et al 1985; Pitkanen et al 1991). In the current study, only healthy rats were recruited and significantly higher levels of NO have been found in PN rats. This indicates that the increased formation of nitric oxide may be due to PN



treatment itself and certain disease condition may have additional effect on the formation of free radicals.

In the current study, a commercially available amino acid mixture, 10%Travasol Amino Acid Injection with electrolytes, was used. It contains a non-essential amino acid, arginine, a substrate of NO synthases. It could be argued that the higher NO, observed in PN rats could be due to the infusion of arginine provided in the amino acid solution. However, arginine-derived NO is a secretory product generated at low concentrations, mainly for cell signaling, in neurons and endothelial cells (Nathan 1992; Stuehr & Griffith 1992; Vane & Botting 1992). Most inducible NO synthases (iNOS), which are present in the endothelial cells, hepatocytes and Kupffer cells are more sensitive to the exposure of endotoxin and cytokines and can be induced to produce high concentrations (Curran et al 1989; Billiar et al 1990; Pittner & Spitzer 1992). Therefore, it is most likely that the higher level of NO found in the liver cytosol was induced by the higher level of endotoxin found in the portal vein, but not caused by the arginine provided in the PN solution.

#### **4.5 Changes of CYP Isoforms After a 7-day PN Treatment *in vitro***

PN has been linked with down-regulation of several CYP isoforms (Earl-Salotti & Charland 1994). After a 7-day PN treatment, Western Blot results suggested that CYP 2C11 (Figure 3.21) was the most suppressed isoform, followed by CYP 3A2 (Figure 3.20). Our results also showed that there were no visible changes for the protein expression of CYP 1A1/2 (Figure 3.20) and the response of CYP 2B1 was inconsistent (Figure 3.21). Unfortunately, Western Blot could only provide qualitative, but not quantitative measurements.



Significant reduction of both total protein content and CYP content in rat liver microsome after PN treatment has been observed (Table 3.3). The CYP content measured in control group in current study was 30% lower compared that of literature values (0.395 nmol/mg vs 0.5 nmol/mg) (**reference**). This could be due to the stress caused by surgery and cannulation during the PN treatment. Previous data (unpublished) from our lab found that the CYP content in rat liver microsome was significantly lower after saline infusion in cannulated rats when compared to that of untreated intact animals.

The kinetic and metabolic data also clearly showed that drug metabolizing enzyme activities were reduced with a 7-day PN treatment. The  $V_{\max}$  values for  $2\alpha$ -hydroxytestosterone production, which was catalyzed by CYP 2C11 in PN animals, were reduced by more than 50% whereas the enzyme activity of CYP2A1/2 was decreased to 66% of the control level. A 25% reduction of the  $V_{\max}$  value of  $6\beta$ -hydroxytestosterone formations, which is catalyzed mainly by CYP 3A1/2, was also observed (Table 3.5). However, this reduction did not reach statistical significance partly due to large standard deviation and relatively small sample size. The other possible reason is that  $6\beta$ -hydroxytestosterone hydroxylation involves multiple isoforms (CYP 3A1/2, 1A1, 2A2) and the observed reduction is the combined results of PN effects on those CYP isoforms.

Our observations are consistent with findings reported by other research groups (Knodell et al 1980; Knodell et al 1984; Knodell et al 1989; Ke et al 1990). Knodell and colleagues (1989) found decreases of 41% in the activity of ethylmorphine demethylase (CYP2C11, 2C2), 73% in the activity of benzphetamine dealkylase (CYP 2C11) and 42% in the activity of erythromycin demethylase (CYP3A2) in PN-treated Sprague-Dawley rats when compared to enterally fed rats. Although there is an overall down-regulation



effect of PN on drug metabolic enzymes, the magnitude of damage reported by different research groups is variable and at times controversial. This could be due to differences of study objectives, design, and more importantly, the composition of PN solution used in different studies.

PN may also have effects on Phase II metabolism. Data suggested that significant decrease of bilirubin conjugate in liver after 5 days of PN infusion (Culebras et al 1993) and an infusion of PN for longer than 10 days markedly decreased hepatic conjugative metabolism (Raftogianis et al 1995). Data also suggested that the transsulfuration pathway that releases inorganic sulfate from dietary cysteine and methionine had been inhibited during PN (Chawla1985). Additionally, sulfoester excretion has been shown to decrease in infants receiving PN that was deficient in inorganic sulfate but not sulfur-containing amino acids (Cole1988). Therefore, it is possible that decreased PAPS availability could be responsible for the observed decrease in sulfation during PN.

The *in vivo* and *in vitro* observations with respect to glucuronidation appear to be contradictory. Experimental results (Raftogianis 1995) have shown that although lipid-free PN inhibited UDP-glucuronosyltransferase activity toward *p*-nitrophenol *in vitro*, the *in vivo* formation clearance of acetaminophen glucuronide was increased in the animals receiving PN for <10 days. Interestingly glucuronidation of acetaminophen *in vivo* has shown no differences to control values in the animals receiving PN for >10 days. The authors stated that different isozymes catalyzing the glucuronidation of acetaminophen and *p*-nitrophenol was responsible for the *in vivo* and *in vitro* differences. Different outcomes due to the length of PN the *in vivo* study may be related to the “latency” associated with glucuronidation activity *in vivo*. Dannenberg et al. (Dannenberg1992) has



observed that a lipid-free diet decreases UDP-glucuronosyl-transferase activity. In the short term, PN may activate the enzyme as reflected by the increase in acetaminophen glucuronidation. Meanwhile, a decrease in the amount of enzyme results owing to the lack of lipid intake. The decline in the amount of enzyme of PN may offset the activating effect of the lipid-free diet, resulting in the apparently normal formation clearance of acetaminophen glucuronide in the rats receiving PN for >10 days.

## **4.6 Effects of Endotoxin, Cytokines and Free Radicals on CYP**

### **Isoforms**

#### **4.6.1 Endotoxin Effects**

It has been reported that endotoxin down-regulates the expression of CYP2C11, a major form of CYP expressed constitutively in male rat liver, at the level of gene transcription (Morgan 1989). It has been shown that mRNAs for 3A2 and 2E1 were down regulated to less than 20% of the control level after endotoxin treatment (Sewer et al 1996). Both the Western Blot and the *in vitro* incubation study results confirmed the suppression of protein expression and lower activities of several CYP isoforms, including CYP2C11, 3A2 and 2B1. The magnitude of suppression is not uniform among different isoforms.

#### **4.6.2 Cytokine Effects**

Cytokines, released from Kupffer cells in the liver, circulating monocytes and macrophages might mediate the effects of endotoxin on CYP expression. Human data (Chen et al 1992; Kurokohchi et al 1992; Morgan et al 1994; Chen et al 1995) have shown that cytokines can act directly on the hepatocyte and cause the depression of CYP-



associated drug metabolism. The role of various cytokines including IL-1 $\beta$ , IL-4, IL-6, TNF- $\alpha$  and IFN- $\gamma$  on the expression and activities of CYP1A2, 2C, 3A, and 2E1 in primary human hepatocyte cultures has been examined (Abdel-Razzak et al 1993). IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were found to be the most potent depressors of CYP enzymes. Both mRNA levels and enzyme activities were depressed, typically by at least 40%. Human hepatoma cell treated with IL-6 also exhibited a significant decrease in the level of CYP3A mRNA (Fukuda et al 1992), suggesting a possible role for IL-6 in the transcriptional regulation of these genes. Direct and/or indirect effects of cytokines on hepatocellular and hepatic drug metabolism have also been suggested from animal data (Sujita et al 1990; Wang et al 1997; Milosevic et al 1999). Chen and colleagues found that intravenous treatment of male rats with IL-6 and TNF- $\alpha$  reduced the activities of hepatic microsomal CYP-dependent monooxygenases to varying degrees (Chen et al 1992).

Further research conducted by Chen and colleagues demonstrated that the expression of CYP 2C11 in male rat primary hepatocyte cultures is suppressed transcriptionally by TNF- $\alpha$ , IL-6 and IL-1 $\beta$  (Chen et al 1995). This agreed with our observation that the 25% reduction of total protein in liver microsome was associated with a 2-3 times higher liver cytosol level of TNF- $\alpha$  and IL-6 after PN-treatment (Figure 3.6 and Table 3.3). The level of individual CYP enzymes in microsomal samples measured using Western Blot also suggested a marked decrease in the expression of CYP2C11, one of the most abundant isoforms in rat liver, and decreased in CYP 3A2 and 2B1 (Figures 3.19 and 3.20). However, similar band intensity was found after incubation with CYP 1A1/2 antibody (Figure 3.20), indicating that TNF- $\alpha$  and IL-6 may not be



potent suppressors of some CYP-dependent enzymes. This set of results is consistent with the findings by the Milosevic group (Milosevic et al 1999). They reported that high TNF- $\alpha$  levels in hepatocytes could result in a strong down-regulation (85%) of CYP isoform CYP2B1, but not CYP1A1 (15%). These results provide evidence that various cytokines have direct effects on the expression and activities of major CYP genes in human and animal hepatocytes.

#### **4.6.3 NO Effects**

Large quantities of nitric oxide interfere with a number of key metabolic enzymes in the body by oxidizing heme or nonhemic iron and iron-sulfur complex (Curran et al 1990; Bredt et al 1991; Moncada et al 1991; Pittner & Spitzer 1992; White & Marletta 1992; Wink et al 1993). CYP isozymes in the liver that metabolize drugs also contain iron, raising the possibility that nitric oxide is a factor in decreasing drug metabolism (Gorodischer et al 1976). *In vitro* data proved that direct exposure of rat hepatic microsomes to nitric oxide inhibits CYP1A1/2, 2B1/2, and 3A1 activities (Khatsenko et al 1993; Wink et al 1993; Muller et al 1994; Stadler et al 1994). Wink and colleagues showed that higher concentration of NO induced by endotoxin impaired CYP-mediated metabolism in isolated hepatic microsomes and the degree of CYP inhibition by endotoxin directly correlated with plasma nitrite levels (Wink et al 1993). Our results agreed with that observation in that higher concentrations of nitric oxide corresponded to lower CYP activities in PN treated rats. This is supported by previous studies (Khatsenko et al 1993; Muller et al 1996) showing inhibition of nitric oxide synthesis partially restored the damage to CYP isoforms 2B1 and 2C11 during endotoxin stimulation. The role of nitric oxide in the



inhibition of CYP isoforms has been correlated with NO generated interferon in human hepatocyte [Donato, 1997 #23].

Depending on lipid emulsions used in parenteral nutrition could go through peroxidation that may be an important feature of oxygen-associated damage. *In vitro* study results (Wanten GJ 1999) suggested neutrophil oxygen radical production is accelerated by mixed medium and long chain triglycerides (Lipofundin) whereas long-chain only triglycerides (Intralipid) no effects.

#### **4.6.4 Other Factors**

One growing area of research in clinical nutrition support has been to modify the conventional parenteral composition and to consider alternative protein and energy sources that may be safer and more effectiveness.

Lipid is a very important component in the PN treatment. However, the beneficial/risk of lipid in PN has been very controversial. Studies link the development of hepatic steatosis during PN treatment to excessive dextrose infusion. Sax and associates (Sax 1986) reported an increased degree of fatty infiltration of the liver in animals receiving a conventional 25% dextrose-based PN solution. Meguid et al found that compared with conventional PN (25% dextrose and 4.25% amino acids), the modified PN (15% dextrose, fat, and 5% amino acids), which 30% of the glucose calories were replaced by fat, significantly reduced the glucose intolerance and abnormal liver function in 88 patients (Meguid MM 1984). Nakagawa and coworkers (Nakagawa 1991) demonstrated that rats treated with hypercaloric PN, especially excess glucose, developed fatty change in the liver. Richardson (Richardson 1975) and Kaminski (Kaminski 1984) proposed that essential fatty acid deficiency might cause fatty infiltration of the liver by



reducing lipoprotein secretion. Stein (Stein 1980) and McDonald (McDonald 1973) demonstrated that correction of essential fatty acid deficiency through lipid administration was associated with improvement in hepatic histology and liver-associated enzymes. But the type of lipid that is infused may influence hepatic detoxification process (Dannenberg AJ 1992). A trial report by Muller et al (1986) showed a higher postoperative mortality rate in patients receiving preoperative lipid-based PN compared with patients receiving lipid-free PN. In a study carried out by Gogos et al (1990), there is no effect on T-cell subsets of malnourished patients after short-term lipid-free PN treatment. Previous data generated from our lab also suggested that lipid-based PN treatment further damaged the CYP enzyme and reduced lidocaine clearance (Zaman). Because the current project is focus on exploring the mechanism of PN effects on CYP enzyme and the hypothesis was that endotoxin triggered the release of cytokine cascade release, the lipid-free PN formula was thus avoid complication.

PN has also been found to impair gut barrier function and enhance endotoxin transport across the gut compared with control animals given pellets enterally (Gonnella 1992). Several studies suggest that PN may depress immunologic function and increase the patient's susceptibility to septic complications. Data from investigations suggest that certain macronutrients may have positive immunomodulating action exclusive of their roles as nutritional substrates when given in pharmacological dosages (Alexander 1990). Adding or substituting some new nutrient such as branched chain amino acid-enriched, glutamine, arginine, and glycine-enriched amino acid or dipeptide formulations in PN solution have shown positive immunomodulating functions (Kirl 1990, Souba 1990).



Arginine is a conditional essential amino acid that is important in the detoxification of ammonia through the urea cycle (Kirl 1990). The positive immunomodulating effects of arginine have been demonstrated in both volunteers and postoperative patients who received pharmacological dosages of arginine with increased T-lymphocyte activity (Daly 1990). Arginine appears to provide greatest immunostimulation when given as a supplement to a complete nutrition formula (Sigal 1992; Daly 1990). The number of adverse effects reported from use of arginine is low. However, most studies to date have infused the supplemental dose of arginine separated from the PN solution, compatible data for supplemental intravenous arginine and PN are lacking.

Glutamine is a nonessential amino acid that functions as an important intermediate for many pathways. Studies of glutamine supplementation in stressed animal models have been associated with greater ability to maintain gut mucosal integrity, presumably by meeting gut demand for glutamine as a preferred fuel (Souba 1990). *In vitro* studies of peripheral human lymphocytes demonstrated a correlation between decreased glutamine concentration and decreased rate of lymphocyte proliferation (Parry-Billings 1990). However, several *in vitro* studies showed glutamine is also a preferred fuel for a number of tumors, therefore use of glutamine supplemented nutrition regimens in tumor-bearing patients is controversial (Souba 1993). Further study is needed to better define optimal doses and contraindications for use of glutamine in different disease states.

Fat serves as an important substrate for critically ill patients such as septic or stressed individual. *In vitro* studies (Cerra 1988) reported conventional long-chain



triglycerides (LCT) in lipid emulsion might impair reticuloendothelial function, impede bacterial clearance, and increase proinflammatory metabolites. The important role of omega-3 fatty acid in health and disease was recognized in 1985 by their beneficial effects on cardiovascular risk factors. Later, dietary manipulations of omega-3 fatty acid have been shown to have a protective effect against tumor growth and provide optimal development in infants (Gottschlich 1992; Lowell 1990).

Although many of these products have indeed found niches in the parenteral nutrition market, their use has been limited to specific patient populations and disease settings.

The reduced enzyme activities in the PN group of animals could be due to poor total body weight gain and lower liver weights. The results in this study showed the gain in body weight and liver weight was within normal range (Table 3.1). This agreed with previous findings in our laboratory when it was shown that PN-treated animals had similar weight as chow-fed animals, but the former exhibited a reduction in drug metabolism (Ke et al 1990). The calorie intake for all animals treated was 330 kcal/kg/day, which is sufficient to provide enough nutrition for normal growth (Ross et al 1983; Ross et al 1984; Knodell et al 1990).

Stress or surgery-related trauma could cause a reduction of drug metabolism but the likelihood of these factors contributing is minimal because a 72 hr recovery period was provided for these animals and this recovery period has been found to minimize metabolic stress in response to trauma induced by surgery (Popp et al 1982). All rats in this study grew normally and gained an average weight of 6.2g/d during the postoperative period.



In our study, the control group rats allowed free access to normal rat diet chow while the PN group received a prescribed volume of PN solution. It is likely that animals in the two treatment groups received different amount of nutrients. However, study carried out by Burgess et al (1987) found that amount of calories (1600 kcal vs 2000 kcal) provided in postoperative patients who received PN for 7 days has no effects on hepatic oxidative functions. Therefore, the observed changes in CYP enzyme after current PN treatment is unlikely caused by different nutrition regimen.

#### **4.7 Changes of Pharmacokinetic Profile of Midazolam *in vivo* After a 7-day PN Treatment**

PN is associated with hepatic dysfunction (Merritt 1986; Klein & Nealon 1988) and therefore may alter hepatic drug elimination (Knodell et al 1980; Ross et al 1983; Knodell et al 1984; Ke et al 1990). However, limited data of PN effects on pharmacokinetics are available in the literature (Hartshorn et al 1979; Moore et al 1981; Kandrotas et al 1988; Koo et al 1990; Jorquera et al 1994; Raftogianis et al 1995; Ronchera-Oms et al 1995) and controversial results have been reported (Hartshorn et al 1979; Ross et al 1983; Jorquera et al 1994). The effects of PN on the pharmacokinetics of midazolam were examined in male rats after a 7-day PN treatment and found that the total body clearance of midazolam was reduced by 40% and the elimination half-life was prolonged from 35 minutes to 49 minutes. There was no change in the value of volume of distribution (Table 3.9). Using the same PN formula, studies by other investigators (Ke et al 1990) have shown that a dextrose and amino acid based PN produced a significant alteration in lidocaine metabolism after a short term PN infusion in rats. Hartshorn and



colleagues observed a decreased antipyrine clearance in rats receiving long-term PN treatment (Hartshorn et al 1979).

It appears that more damage of CYP 3A2 mediated reaction was observed *in vivo* when MDZ was used as a substrate when compared to that of testosterone *in vitro* (Table 3.5). However, if one takes the 25% reduction of the  $V_{\max}$  value for the formation of 6 $\beta$ -hydroxytestosterone and the reduction of 20% to 25% CYP content into account, the *in vitro* and *in vivo* results would be comparable. This PN-induced damage on CYP 3A2 was also confirmed with the Western Blot results (Figure 3.20) where lighter and thinner bands were observed in samples collected from PN rats although the difference can not be quantitatively determined. *In vitro* metabolic study of MDZ should be performed in the future to examine the potential *in vivo* and *in vitro* correlation.

Raftogianis and colleagues demonstrated a 23% decrease of total clearance of acetaminophen in rats after a 10-day PN treatment, suggesting that PN also depress phase II conjugative metabolism (Raftogianis et al 1995). Along with our results, all findings support that PN treatment could damage liver oxidative enzymes and reduced drug clearance *in vivo* in an animal model.

Linear Pharmacokinetics was observed for MDZ in a dose-dependent study (Bornemann et al 1985) where escalated doses up to 15 mg/kg were given. This indicated that pharmacokinetic parameter obtained after a 3 mg/kg iv dose falls in the linear range. MDZ has a medium to high hepatic extraction ratio. Significant reduction (40%) of systemic clearance of MDZ was measured in PN treated animals. The increase in AUC would be expected to be much higher if MDZ was to be given only to patients who are receiving PN.



Data from human studies are more complicated and controversial. Jorquera and coworkers found that antipyrine clearance was increased by 61% in surgical patients maintained on hypocaloric PN (Jorquera et al 1994). Kandrotas and colleagues also reported an increased clearance of aminophylline after 8 weeks of PN treatment in premature neonates (Kandrotas et al 1988). On the other hand, other investigators found that a PN formulation containing 3.75% amino acid and 10% dextrose didn't significantly alter the pharmacokinetic parameters of ampicillin in seven healthy volunteers (Koo et al 1990). Also, no changes in cimetidine pharmacokinetic profile were discovered when this drug was coinfused with PN in patients with gastric hypersecretion (Moore et al 1981). The inconsistency in human data could be due to a number of reasons, such as the different PN formulae being used among the different research groups, the impact of patients' disease status and the small numbers of subjects enrolled. All these factors make the interpretation of PN effects on the drug metabolism in human difficult. Careful and unified study designs are needed in the future.

## **4.8 Conclusion**

In the past decade, PN effects on drug metabolism have been intensively studied. However, the underlying mechanisms to explain these effects remained unclear. In this study, a possible mechanism towards a better understanding of the relationship among bacterial translocation, cytokines, and their possible effects on the activity of liver drug metabolic enzymes have been proposed.



In this project, it was found that after a 7-day continuous PN infusion to rats, a higher level of endotoxin was observed in the portal vein, but not in peripheral veins of PN rats, providing evidence of bacterial translocation during PN treatment.

The studies now reported also revealed that higher levels of TNF- $\alpha$  and IL-6 were present in liver cytosol of PN-treated rats. This could be triggered by higher levels of endotoxin in the portal vein during PN infusion, since endotoxin is a potent stimulant of numerous cytokines. A high nitrite level in liver cytosol of PN treated rats was also detected. Since nitrite is an indicator of nitric oxide, the results clearly suggest that higher concentration of cytokines measured in the PN treated rats is partially linked to the elevation of free radicals in the experimental animals.

The *in vitro* studies confirmed that after a 7-day PN treatment, the protein expression and activities of a number of important CYP isoforms, including CYP 2C11, 3A2, 2A, have been suppressed, while others including CYP 1A1/2 and 2B1 have shown no response to the PN treatment. The mechanisms responsible for these selective changes in the synthesis and function of individual CYP remain to be elucidated

In the *in vivo* study, it is demonstrated that total body clearance of midazolam, a substrate of CYP 3A2 in rodent, has been dramatically reduced after continuous PN infusion. This could be due to the lower activity of CYP 3A2 caused by PN.

In the literature, researchers have suggested that bacterial translocation occurred during PN treatment. PN has also been linked with down-regulations of CYP isozymes. Meanwhile, numerous reports indicate that both cytokines and free radicals are strong suppressors of CYP isoforms. However, those pieces of information have been interrelated when attempts were made to explore the potential underlying mechanisms. In



this study, for the first time, it has been possible to produce links among the effects of PN, bacterial translocation, cytokines and free radicals on drug metabolism. Based on this set of results, it can be postulated that a) bacterial translocation occurred during PN; therefore bacteria and endotoxin enters the portal system; b) endotoxin from the gut triggers the release of several cytokines and free radicals, such as TNF- $\alpha$ , IL-6, and nitric oxide, in the liver; c) cytokines and free radical selectively suppress expression of certain CYP isozymes and lower their activities; d) the damaged CYP enzymes alter the pharmacokinetics of some drugs, such as midazolam.

#### **4.9 Future work**

There are number of studies which can be performed in the future such that the effect Of PN on drug meatoblism can better be evaluated.

1. Conduct *in vitro* metabolic study of MDZ after PN treatment to better correlate *in vivo* and *in vitro* results.
2. Study the time-course of cytokine and free radical release during PN treatment.
3. Further explore PN effects on Phase II metabolism both *in vivo* and *in vitro*.
4. Study the effects of macronutrients on the function of Phase I and Phase II metabolism.



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